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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,253,647**, on December 10, 1998, by **HSC RESEARCH AND DEVELOPMENT LIMITED PARTNERSHIP.**, assignee of Jeffrey L. Wrana, for "Smad Binding Proteins".

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Smad BINDING PROTEINS

Field of the Invention

The invention relates to a family of proteins, the SARA proteins, which bind to receptor-regulated Smad proteins and are involved in appropriate localization of these Smad proteins for receptor activation.

Background of the Invention

The Transforming Growth Factor-beta (TGFβ) superfamily, whose members include TGFβs, activins and bone morphogenetic proteins (BMPs), have wide ranging effects on cells of diverse origins (Attisano and Wrana, 1998; Heldin et al., 1997; Kretzschmar and Massagué, 1998). Signaling by these secreted factors is initiated upon interaction with a family of cell-surface transmembrane serine/threonine kinases, known as type I and type II receptors. Ligand induces formation of a typeI/typeII heteromeric complex which permits the constitutively active type II receptor to phosphorylate, and thereby activate, the type I receptor (Wrana et al., 1994). This activated type I receptor then propagates the signal to a family of intracellular signaling mediators known as Smads (Attisano and Wrana, 1998; Heldin et al., 1997; Kretzschmar and Massagué, 1998).

The first members of the Smad family identified in invertebrates were the *Drosophila* MAD and the C. *elegans* sma genes (sma-2, sma-3 and sma-4; Savage et al., 1996; Sekelsky et al., 1995). Currently, the family includes additional invertebrate Smads, as well as nine vertebrate members, Smad1 through 9 (Attisano and Wrana, 1998; Heldin et al., 1997; Kretzschmar and Massagué, 1998). Smad proteins contain two conserved amino (MH1) and carboxy (MH2) terminal regions separated by a more divergent linker region. In general, Smad proteins can be subdivided into three groups; the receptor-regulated Smads, which include Smad 1, 2, 3, 5 and 8, Mad, sma-2 and sma-3; the common Smads, Smad4 and Medea, and the antagonistic Smads, which include Smad6, 7

and 9, DAD and daf-3 (Heldin et al., 1997; Nakayama et al., 1998; Patterson et al., 1997).

Numerous studies with vertebrate Smad proteins have provided insights into the differential functions of these proteins in mediating signaling. Receptor-regulated Smads are direct substrates of specific type I receptors and the proteins are phosphorylated on the last two serines at the carboxy-terminus within a highly conserved SSXS motif (Abdollah et al., 1997; Kretzschmar et al., 1997; Liu et al., 1997b; Macias-Silva et al., 1996; Souchelnytskyi et al., 1997). Interestingly, Smad2 and Smad3 are substrates of TGFβ or activin receptors and mediate signaling by these ligands (Liu et al., 1997b; Macias-Silva et al., 1996; Nakao et al., 1997a), whereas Smad1, 5 and 8 appear to be targets of BMP receptors and thereby propagate BMP signals (Chen et al., 1997b; Hoodless et al., 1995; Kretzschmar et al., 1997; Nishimura et al., 1998). Once phosphorylated, these Smads bind to the common Smad, Smad4, which lacks the carboxy-terminal phosphorylation site and is not a target for receptor phosphorylation (Lagna et al., 1996; Zhang et al., 1997). Heteromeric complexes of the receptor-regulated Smad and Smad4 translocate to the nucleus where they function to regulate the transcriptional activation of specific target genes. The antagonist Smads, Smad6, 7 and 9 appear to function by blocking ligand-dependent signaling by preventing access of receptor-regulated Smads to the type I receptor or possibly by blocking formation of heteromeric complexes with Smad4 (reviewed in Heldin et al., 1997).

Analysis of the nuclear function of Smads has demonstrated that Smads can act as transcriptional activators and that some Smads, including *Drosophila* Mad, and the vertebrate Smad3 and Smad4, can bind directly to DNA, albeit at relatively low specificity and affinity (Dennler et al., 1998; Kim et al., 1997; Labbé et al., 1998; Yingling et al., 1997; Zawel et al., 1998).

Localization of Smads is critical in controlling their activity and Smad phosphorylation by the type I receptor regulates Smad activity by inducing nuclear accumulation (Attisano and Wrana, 1998; Heldin et al., 1997; Kretzschmar and Massagué, 1998). However, little is known about how Smad

localization is controlled prior to phosphorylation and how this might function in modulating receptor interactions with its Smad substrates.

Summary of the Invention

Smad proteins (Smads) transmit signals from transmembrane ser/thr kinase receptors to the nucleus. Mammalian and non-mammalian proteins have been identified which interact directly with Smads and are designated the Smad Anchor for Receptor Activation or SARA proteins.

The invention provides cDNA sequences encoding this previously undescribed family of SARA proteins which bind to receptor-regulated Smad proteins and ensure appropriate localization of these Smad proteins for activation by a Type I receptor of a TGFβ, activin or BMP signaling pathway.

For example, TGFβ signaling induces dissociation of Smad2 or Smad3 from a SARA protein with concomitant formation of Smad2/Smad4 or Smad3/Smad4 complexes and nuclear translocation. In the absence of signaling, SARA functions to recruit a particular Smad (eg. Smad2 or Smad3) to distinct subcellular sites in the cell and interacts with the TGFβ superfamily receptor complex in cooperation with the particular receptor regulated Smad. Mutations in hSARA1 that cause mislocalization of Smad2, and interfere with receptor association, inhibit receptor-dependent transcriptional responses, indicating that regulation of Smad localization is essential for TGFβ superfamily signaling. The invention provides a novel component of the signal transduction pathway that functions to anchor Smads to specific subcellular sites for activation by the Type I receptor of the TGFβ, activin or BMP signaling pathways.

The SARA proteins are characterised by the presence of three domains, a double zinc finger or FYVE domain responsible for the subcellular localisation of the SARA protein or SARA-Smad complex, a Smad-binding domain which mediates the interaction or binding of one or more species of Smad protein and a carboxy terminal domain which mediates association with the TGFβ superfamily receptor. The FYVE domain may bind phosphatidyl inositol-3-phosphate.

In accordance with one embodiment, the invention provides isolated polynucleotides comprising nucleotide sequences encoding SARA proteins.

In accordance with a further series of embodiments, the invention provides an isolated polynucleotide selected from the group consisting of

(a) a nucleotide sequence encoding a human SARA protein;

(b) a nucleotide sequence encoding a mammalian SARA protein;

(c) a nucleotide sequence encoding a non-mammalian SARA protein;

(d) a nucleotide sequence encoding the human SARA amino acid sequence of Table 2 (hSARA1: Sequence ID NO:2);

(e) a nucleotide sequence encoding the human SARA amino acid sequence of Table 4 (hSARA2: Sequence ID NO:4);

(f) a nucleotide sequence encoding the *Xenopus* SARA amino acid sequence of Table 6 (XSARA1: Sequence ID NO:6);

(g) a nucleotide sequence encoding the *Xenopus* SARA amino acid sequence of Table 8 (XSARA2: Sequence ID NO:8).

In accordance with a further embodiment, the invention provides the nucleotide sequences of Table 1 (human SARA1 or hSARA1), Table 3 (human SARA2 or hSARA2), Table 5 (Xenopus SARA1 or XSARA1) and Table 7 (Xenopus SARA2 or XSARA2).

In accordance with a further embodiment, the invention provides recombinant vectors including the polynucleotides disclosed herein and host cells transformed with these vectors.

The invention further provides a method for producing SARA proteins, comprising culturing such host cells to permit expression of a SARA protein-encoding polynucleotide and production of the protein.

The invention also includes polynucleotides which are complementary to the disclosed nucleotide sequences, polynucleotides which hybridize to these sequences under high stringency and degeneracy equivalents of these sequences.

In accordance with a further embodiment, the invention provides antisense molecules which may be used to prevent expression of a SARA protein. Such antisense molecules can be synthesised by methods known to those skilled in the art and include phosphorothioates and similar compounds.

The invention further includes polymorphisms and alternatively spliced versions of the disclosed SARA genes and proteins wherein nucleotide or amino acid substitutions or deletions do not substantially affect the functioning of the gene or its encoded protein.

The invention also enables the identification and isolation of allelic variants or homologues of the described SARA genes, and their corresponding proteins, using standard hybridisation screening or PCR techniques.

The invention provides a method for identifying allelic variants or homologues of the described SARA genes, comprising

choosing a nucleic acid probe or primer capable of hybridizing to a SARA gene sequence under stringent hybridisation conditions;

mixing the probe or primer with a sample of nucleic acids which may contain a nucleic acid corresponding to the variant or homologue; and

detecting hybridisation of the probe or primer to the nucleic acid corresponding to the variant or homologue.

In accordance with a further embodiment, the invention provides fragments of the disclosed polynucleotides, such as polynucleotides of at least 10, preferably 15, more preferably 20 consecutive nucleotides of the disclosed polynucleotide sequences. These fragments are useful as probes and PCR primers or for encoding fragments, functional domains or antigenic determinants of SARA proteins.

In accordance with a further embodiment, the invention provides substantially purified SARA proteins, including the proteins of Table 2 (hSARA1), Table 4 (hSARA2), Table 6 (XSARA1) and Table 8 (XSARA2).

In accordance with one embodiment, a SARA protein has a FYVE domain, a Smad binding domain (SBD) and an amino acid sequence having at least 50% overall identity with the amino acid sequence of hSARA1 (Sequence ID NO:2).

In accordance with a preferred embodiment, a SARA protein has a FYVE domain having at least 65% identity of amino acid sequence with the FYVE domain of hSARA1 and a C-terminal sequence of 550 consecutive amino acids which have at least 50% identity with the C-terminal 550 amino acid residues of hSARA1.

In accordance with a more preferred embodiment, a SARA protein has a FYVE domain having at least 65% identity of amino acid sequence with the FYVE domain of hSARA1 and wherein the portion of the SBD corresponding to amino acid residues 721 to 740 of hSARA1 has at least 80% identity with that portion of hSARA1.

The invention further provides a method for producing antibodies which selectively bind to a SARA protein comprising the steps of

administering an immunogenically effective amount of a SARA immunogen to an animal;

allowing the animal to produce antibodies to the immunogen; and

obtaining the antibodies from the animal or from a cell culture derived therefrom.

The invention further provides substantially pure antibodies which bind selectively to an antigenic determinant of a SARA protein. The antibodies of the invention include polyclonal antibodies, monoclonal antibodies and single chain antibodies.

The invention includes analogues of the disclosed protein sequences, having conservative amino acid substitutions therein. The invention also includes fragments of the disclosed protein sequences, such as peptides of at least 6, preferably 10, more preferably 20 consecutive amino acids of the disclosed protein sequences.

The invention further provides polypeptides comprising at least one functional domain or at least an antigenic determinant of a SARA protein.

In accordance with a further embodiment, the invention provides peptides which comprise SARA protein Smad binding domains and polynucleotides which encode such peptides.

In accordance with a further embodiment, the invention provides a Smad binding domain peptide selected from the group consisting of

(a) SASSQSPNPNNPAEYCSTIPPLQQAQASGALSSPPPTVMVPVGV LKHPGAEVAQPREQRRVWFADGILPNGEVADAAKLTMNGTSS; and

(b) amino acids 589 to 672 of the XSARA1 sequence of Table 9.

The invention includes fragments and variants of these Smad binding domain peptides which retain the ability to bind a Smad protein.

In accordance with a further embodiment, the invention provides peptides which comprise SARA protein FYVE domains and polynucleotides which encode such peptides.

In accordance with a further embodiment, the invention provides a FYVE domain peptide selected from the group consisting of

(a) amino acids 587 to 655 of the hSARA1 sequence of Table 9;

(b) amino acids 510 to 578 of the XSARA1 sequence of Table 9; and

(c) the consensus amino acid sequence of Table 10.

The invention includes fragments and variants of these FYVE domain peptides which retain the function of the parent peptide.

In accordance with a further embodiment, the invention provides peptides which comprise SARA protein TGFβ receptor interacting domains and polynucleotides which encode such peptides.

In accordance with a further embodiment, the invention provides a TGFβ receptor interacting domain peptide comprising amino acids 751 to 1323 of the hSARA1 sequence of Table 9.

The invention includes fragments and variants of these TGFβ receptor binding domain peptides which retain the binding ability of the parent peptide.

The invention further provides methods for modulating signaling by members of the TGFβ superfamily which signal through pathways which involve a SARA protein.

Modulation of signaling by a TGFβ superfamily member through such a pathway may be effected, for example, by increasing or reducing the binding of the SARA protein involved in the pathway with its binding partner.

In accordance with a further embodiment, TGFβ superfamily signaling, including TGFβ signaling, by a pathway involving a SARA protein described herein may be modulated by modulating the binding of the SARA protein to a Smad binding partner, by modulating the binding of its FYVE domain to its binding partner or by modulating the binding of the SARA protein to a TGFβ superfamily receptor, such as the TGFβ receptor.

For example, the binding of a SARA protein to a Smad binding partner may be inhibited by a deletion mutant of the protein lacking either the SBD domain or the FYVE domain or by the SARA protein Smad binding domain peptides or FYVE domain peptides described herein, and effective fragments or variants thereof. The binding of a SARA protein to a TGFβ superfamily receptor may be inhibited by a deletion mutant of the protein lacking a C terminal portion or by the SARA protein TGFβ receptor binding domain peptides described herein, and effective fragments and variants thereof.

In accordance with a further embodiment, TGFβ superfamily signaling, including TGFβ signaling, by a pathway involving a SARA protein may be modulated by modulating the binding of the SARA protein FYVE domain to phosphatidyl inositol-3-phosphate, by increasing or decreasing the availability of phosphatidyl inositol-3-phosphate or by administration of agonists or antagonists of phosphatidyl inositol-3-phosphate kinase.

The invention also provides a method of modulating a TGFβ superfamily signaling pathway involving phosphatidyl inositol-3-phosphate, including a TGFβ signaling pathway, by increasing or decreasing the availability of SARA protein or by modulating the function of SARA protein.

The invention further provides methods for preventing or treating diseases characterised by an abnormality in a TGFβ superfamily member signaling pathway which involves a SARA protein, by modulating signaling in the pathway, as described above.

TGFβ signaling is important in wound healing, and excessive signaling is associated with scarring, with arthritis and with fibrosis in numerous diseases, including fibrosis of the liver and kidney. TGFβ signaling is also involved in

modulating inflammatory and immune responses and can contribute to tumour progression.

The invention thus provides methods for modulating TGFβ-dependent cell proliferation or fibrogenesis.

The BMP signaling pathways are important in tissue morphogenesis and in protecting tissues and restoring or regenerating tissues after tissue damage, for example in bone, kidney, liver and neuronal tissue (see, for example, (Reddy, A.H. (1998), *Nature Biotechnology*, v. 16, pp. 247-252).

The invention further provides methods for modulating BMP-dependent phenotypic marker expression by modulating the interactions of SARA proteins involved in these BMP signaling pathways.

In accordance with a further embodiment, modified versions of a SARA protein may be provided as dominant-negatives that block TGFβ superfamily signaling. These modified versions of SARA could, for example, lack the Smad binding domain and thereby prevent recruitment of Smad or could lack the FYVE domain and thereby inhibit signaling by interfering with translocation.

These modified versions of SARA may be provided by gene therapy, for example using transducing viral vectors. Expression may be driven by inclusion in the vector of a promoter specific for a selected target cell type. Many examples of such specific promoters are known to those skilled in the art.

In a further embodiment, a normal version of a SARA protein such as hSARA1 could be provided by gene therapy to restore function in a disease wherein SARA is mutated or non-functional.

In a further embodiment, the invention provides a pharmaceutical composition comprising a purified SARA protein as active ingredient.

In accordance with a further embodiment, the invention provides non-human transgenic animals and methods for the production of non-human transgenic animals which afford models for further study of the SARA system and tools for screening of candidate compounds as therapeutics. For example, knock out animals, such as mice, may be produced with deletion of a SARA gene.

These animals may be examined for phenotypic changes and used to screen candidate compounds for effectiveness to reverse these changes.

In a further example, transgenic animals may be produced expressing a dominant negative mutant of a SARA protein, as described above, either generally or in specific targeted tissues.

The invention provides many targets for the development of small molecule drugs, including peptides and peptidomimetic drugs, to interfere with the interaction of the various binding partners described herein and thereby modulate signaling by members of the TGFβ superfamily, including TGFβ and BMPs.

The invention further provides methods for screening candidate compounds to identify those able to modulate signaling by a member of the TGFβ superfamily through a pathway involving a SARA protein.

For example, the invention provides screening methods for compounds able to bind to a SARA protein which are therefore candidates for modifying the activity of the SARA protein. Various suitable screening methods are known to those in the art, including immobilization of a SARA protein on a substrate and exposure of the bound SARA protein to candidate compounds, followed by elution of compounds which have bound to the SARA protein. The methods used to characterise the binding interactions of the SARA proteins disclosed herein, as fully described in the examples herein, may also be used to screen for compounds which are agonists or antagonists of the binding of a SARA protein.

This invention also provides methods of screening for compounds which modulate TGFβ superfamily signaling by detecting an alteration in the phosphorylation state of a SARA protein.

In accordance with a further embodiment, the invention provides a method for reducing or preventing TGFβ, activin or BMP signaling by inhibiting the activity of SARA. SARA activity may be inhibited by use of an antisense sequence to the SARA gene or by mutation of the SARA gene.

Summary of the Drawings

Certain embodiments of the invention are described, reference being made to the accompanying drawings, wherein:

Figure 1 (top panel) shows interaction of full length hSARA1 with bacterially expressed Smads. Full length SARA protein was produced in an *in vitro* transcription/translation system in the presence of [^{35}S]methionine and was incubated with glutathione-sepharose beads coated with bacterially-expressed GST fusion proteins of the indicated Smads or Smad2 subdomains. Bound material was resolved by SDS-PAGE and visualized by autoradiography. Migration of full length hSARA1, and a translation product that initiates from an internal methionine located upstream of the Smad binding domain (asterisk) are indicated. The presence of approximately equivalent amounts of GST fusion proteins was confirmed by SDS-PAGE and coomassie staining of a protein aliquot (bottom panel).

Figure 2 shows interaction of hSARA with Smads in mammalian cells. COS cells were transfected with Flag-tagged hSARA1 (Flag-SARA) either alone or together with the indicated Myc-tagged Smad constructs. For Smad6, an alternative version lacking the MH1 domain was used (Topper et al., 1997). Cell lysates were subjected to an anti-Flag immunoprecipitation and coprecipitating Smads detected by immunoblotting with anti-Myc antibodies. The migration of anti-Flag heavy and light chains (IgG) are marked. To confirm efficient expression of hSARA1 and the Smads, aliquots of total cell lysates were immunoblotted with the anti-Flag and anti-Myc antibodies (bottom panel). The migrations of hSARA1 and the Smads are indicated.

Figures 3-6 show immunoblots of lysates from COS cells transiently transfected with various combinations of Flag or Myc-tagged hSARA1, wild type (WT) or mutant (2SA) Myc or Flag-tagged Smad2, Smad4/HA and wild type (WT) or constitutively active (A) TβRI/HA, cell lysates being subjected to immunoprecipitation with anti-Flag or anti-Myc antibodies, as indicated. Confirmation of protein expression was performed by immunoblotting total cell

lysates prepared in parallel for the indicated tagged protein (totals, bottom panels).

Figure 3: Transfected cells were metabolically labelled with [^{32}P]PO_4 and cell lysates subjected to immunoprecipitation with anti-Flag antibodies for visualization of hSARA1 phosphorylation (top panel) or with anti-Myc antibodies for Smad2 phosphorylation (middle panel). Immunoprecipitates were resolved by SDS-PAGE and visualized by autoradiography. The migrations of hSARA1 and Smad2 are indicated.

Figure 4: Lysates from transiently transfected COS cells were subjected to immunoprecipitation with anti-Flag antibodies and Smad2 bound to hSARA1 was analyzed by immunoblotting with anti-Myc antibodies (IP: α-flag; blot: α-Myc).

Figure 5: Lysates from transiently transfected COS cells were subjected to immunoprecipitation with anti-Flag antibodies and Smad2 bound to hSARA1 was analyzed by immunoblotting with anti-Myc antibodies (IP: α-flag, blot: α-Myc). Partial dissociation of hSARA1/Smad2 complexes induced by TGFβ signaling was enhanced by expression of Smad4.

Figure 6: Cell lysates from transiently transfected COS cells were subjected to immunoprecipitation with anti-Flag antibodies directed towards Smad2. Immunoprecipitates were then immunoblotted using anti-Myc or anti-HA antibodies which recognize hSARA1 or Smad4, respectively. Coprecipitating SARA (α-myc blot) and Smad4 (α-HA blot) are indicated.

Figure 7, panels A to E, shows photomicrographs of Mv1Lu cells transiently transfected with various combinations of Flag-Smad2, Myc-hSARA1, and constitutively active TβRI (TβRI*) as indicated (Tx). hSARA was visualized with the polyclonal Myc A14 antibody and Texas-Red conjugated goat-anti-rabbit IgG (red) and Smad2 was detected with an anti-Flag M2 monoclonal antibody followed by FITC-conjugated goat anti-mouse IgG (green). The subcellular localization of the expressed proteins was visualized by immunofluorescence and confocal microscopy.

Panels A, B, C, Mv1Lu cells singly transfected with hSARA1 (A) or Smad2 (B) are shown. Cotransfection of Smad2 with the constitutively active TβRI (TβRI*) results in its accumulation in the nucleus (C).

Panel D, Mv1Lu cells were transfected with hSARA1 and Smad2 and the localization of hSARA1 (red, left photo) and Smad2 (green, centre photo) is shown. Colocalization of SARA and Smad2 is shown (right photo) and appears as yellow.

Panel E, Mv1Lu cells were transfected with hSARA1, Smad2 and activated TβRI (TβRI*) and the localization of hSARA (red, left photo) and Smad2 (green, centre photo) is shown. Colocalization of SARA and Smad2 is indicated (right photo). Note the shift to an orangy-red colour in the punctate spots and an intensification of Smad2 nuclear staining, indicative of dissociation of Smad2 from SARA and nuclear translocation.

Figure 7, panel F, shows photomicrographs of Mv1Lu cells stained with rabbit, polyclonal anti-SARA antibody (left photo, green), goat, polyclonal anti-Smad 2/3 antibody (centre photo, red) and with both antibodies (right photo, yellow), showing co-localization of hSARA1 and Smad2.

Figure 8A shows photomicrographs of Mv1Lu cells transfected with either hSARA1 alone (panel i), TβRII alone (panel ii) or hSARA1 and TβRII together (panel iii), then treated with TGFβ and the localization of hSARA1 (red) and TβRII (green) determined by immunofluorescence and confocal microscopy. In cells coexpressing hSARA1 and TβRII, superimposing the staining revealed colocalization of the proteins as indicated by yellow staining in panel iii.

Figure 8B shows affinity labelling of COS cells transiently transfected with various combinations of Flag-hSARA1, Myc-Smad2, wild type (WT) TβRII and either wild type or kinase-deficient (KR) versions of TβRI. Cells were affinity-labelled with [^{125}I]TGFβ and lysates immunoprecipitated with anti-Flag antibodies. Coprecipitating receptor complexes were visualized by SDS-PAGE and autoradiography. Equivalent receptor expression was confirmed by visualizing aliquots of total cell lysates (bottom panel).

Figure 9A shows COS cells transiently transfected with wild type TβRII and kinase-deficient TβRI and various combinations of wild type Flag-hSARA1 (WT), a mutant version lacking the Smad2 binding domain (ΔSBD) and Myc-Smad2. The amount of receptor bound to SARA was determined by anti-Flag immunoprecipitation followed by gamma counting. Data is plotted as the average of three experiments ± S.D. Protein expression was analyzed by immunoblotting aliquots of total cell lysates and the results from a representative experiment are shown (bottom panel).

Figure 9B shows COS cells transiently transfected with wild type TβRII and kinase-deficient TβRI and Flag-tagged wild type (WT) or mutant versions of hSARA1 with (black bars) or without (open bars) Myc-Smad2. The amount of receptor bound to hSARA1 was determined by anti-Flag immunoprecipitation followed by gamma counting. Protein expression was analyzed by immunoblotting aliquots of total cell lysates (bottom panel).

Figure 10 is a schematic representation of mutant versions of SARA. The FYVE domain (shaded bar) and the Smad binding domain, SBD (striped bar), are indicated. COS cells transiently transfected with Flag-hSARA1 and Myc-Smad2 were immunoprecipitated with anti-Flag antibodies followed by immunoblotting with anti-Myc antibodies. The presence (+) or absence (-) of a hSARA1/Smad2 interaction is indicated (Smad2 interaction). Mutants used for the subsequent localization study are marked on the left (i-vi).

Figure 11A shows an immunoblot of lysates from COS cells expressing Flag-tagged Smad2 or Smad3 incubated with GST alone or with GST-hSARA1 (665-750), which corresponds to the SBD; bound proteins were immunoblotted using anti-Flag antibodies. The presence of Smad2 and Smad3 bound to GST-hSARA1 (665-750) is indicated.

Figure 11B shows an immunoblot of lysates, from COS cells expressing Flag-tagged Smad2 together with wild type (WT) or activated (A) type I receptor, incubated with GST-hSARA1 (665-750) (GST-SBD) and immunoblotted with anti-Flag antibodies. The expression levels of Smad2, each receptor and GST-

hSARA1 (665-750) were determined by immunoblotting aliquots of total cell lysates.

Figure 12 shows the subcellular localization of hSARA1 mutants. Mv1Lu cells were transiently transfected with wild type (panel i) or mutant versions of Flag-hSARA1 (panels ii-viii, as marked on the left in Figure 10). Proteins were visualized by immunofluorescence and confocal microscopy using a monoclonal anti-Flag M2 monoclonal antibody followed by FITC-conjugated goat anti-mouse IgG.

Figure 13 shows photomicrographs of Mv1Lu cells transiently transfected with mutant versions of Myc-hSARA1 and Flag-Smad2 (panel A) or with wild type Myc-hSARA1, HA-Smad2 and mutant versions of hSARA1 (panel B). Protein subcellular localization was visualized by immunofluorescence and confocal microscopy. hSARA1 was visualized with the polyclonal Myc A14 antibody and FITC-conjugated goat anti-rabbit IgG (green), while Smad2 was detected with monoclonal antibodies followed by Texas Red-conjugated goat anti-mouse IgG (red). In B, overlaying the images reveals mislocalization of Smad2 as green speckles of SARA over red, diffuse Smad2 staining (panels ii and iii) and colocalization of hSARA1 and Smad2 appears as yellow spots (panels i and iv).

Figure 14 shows luciferase activity of Mv1Lu cells transfected with 3TP-lux alone or together with the indicated amounts of wild type (WT) or mutant (Δ1-664 or Δ1-704) versions of hSARA1 and incubated in the presence (black bars) or absence (open bars) of TGFβ. Luciferase activity was normalized to β-galactosidase activity and is plotted as the mean $\pm$S.D. of triplicates from a representative experiment.

Figure 15 shows luciferase activity of HepG2 cells transfected with ARE-Lux alone (v), or ARE-Lux and FAST2 alone or together with the indicated amounts of wild type (WT) or mutant versions of hSARA1. Transfected cells were incubated in the presence (black bars) or absence (open bars) of TGFβ and luciferase activity was normalized to β-galactosidase activity and is plotted as the mean $\pm$S.D. of triplicates from a representative experiment.

Figure 16 shows a Northern blot of expression of hSARA1 (upper panel) and Smad2 (lower panel) in the indicated tissues.

Figure 17 shows an immunoblot of a HepG2 lysate immunoprecipitated (IP) with preimmune serum (PI), anti-hSARA1 polyclonal antibody (SARA) with and without pretreatment with TGFβ (- and +), or N19 anti-Smad2/3 antibody (S2), followed by immunoblotting with an anti-Smad2 antibody. The migration position of Smad2 is indicated (Smad2).

Figure 18 shows a diagram of a model of the interaction of a SARA protein with a receptor regulated Smad, as exemplified by the interaction of hSARA1.

Detailed Description of the Invention

This invention provides a family of proteins that play key roles in TGF-β, activin and bone morphogenetic protein (BMP) signal transduction pathways. In particular, the proteins of this family interact with specific Smad proteins to modulate signal transduction. These proteins are therefore designated as "Smad Anchor for Receptor Activation" or "SARA" proteins. SARA proteins are characterised by three distinct domains (1) a double zinc finger or FYVE domain responsible for the subcellular localization of the SARA protein or SARA-Smad complex, possibly through its association with PtdIns(3)P, (2) a Smad binding domain ("SBD") which mediates the interaction or binding of one or more species of Smad protein with the particular member of the SARA family and (3) a carboxy terminal domain which mediates interaction of SARA with members of the TGFβ superfamily of receptors.

FYVE domains have been identified in a number of unrelated signaling molecules that include FGD1, a putative guanine exchange factor for Rho/Rac that is mutated in faciogential dysplasia, the HGF receptor substrate Hrs-1 and its homolog Hrs-2, EEA1, a protein involved in formation of the early endosome and the yeast proteins FAB1, VPS27 and VAC1 (reviewed in Wiedemann and Cockcroft, 1998). Recently, analysis of a number of FYVE domains from yeast and mammals has revealed that this motif binds phosphatidyl inositol-3-

phosphate (PtdIns(3)P) with high specificity and thus represents a novel signaling module that can mediate protein interaction with membranes (Burd and Emr, 1998; Gaullier et al., 1998; Patki et al., 1998; Simonsen et al., 1998; Wiedemann and Cockcroft, 1998). Comparison of the FYVE domains from the vertebrate proteins with that from SARA revealed extensive conservation of residues throughout the domain (Table 10). Thus, SARA contains a FYVE domain that may function to bind PtdIns(3)P, which has been implicated in intracellular vesicle transport.

For example, deletion of the FYVE domain in hSARA1 causes mislocalization of Smad2 or Smad3, interferes with TGFβ receptor interaction and inhibits TGFβ-dependent transcriptional responses.

Thus, the SARA proteins of the invention define a component of TGFβ superfamily signaling that fulfills an essential role in anchoring receptor regulated Smads to specific subcellular domains for activation by a TGFβ superfamily receptor.

Cloned DNA coding sequences and corresponding amino acid sequences for representative human and Xenopus SARA protein family members are shown in Tables 1 and 2 - human SARA1 (hSARA1), Tables 3 and 4 - human SARA2 (hSARA2), Tables 5 and 6 - Xenopus SARA1 (XSARA1) and Tables 7 and 8 - Xenopus SARA2 (XSARA2).

Table 9 shows a comparison of the amino acid sequences of XSARA1 and hSARA1. Identical residues (dark grey) and conservative changes (light grey), the FYVE domain (solid underline) and the Smad binding domain (dashed underline) are indicated. The sequences in XSARA1 used to design degenerate PCR primers for identifying hSARA1 are shown (arrows). The amino-terminal end of the partial Xenopus cDNA obtained in the expression screen is marked (asterisk).

The human SARA of Tables 1 and 2, identified as described in Example 2, regulates the subcellular localization of Smad2 and Smad3 and recruits these Smads into distinct subcellular domains. This SARA also interacts with TGFβ receptors and TGFβ signaling induces dissociation of Smad2 or Smad3 from the

SARA protein with concomitant formation of Smad2/Smad4 complexes and nuclear translocation.

Table 10 shows alignment of the amino acid sequences of the FYVE domains from hSARA1, XSARA1, KIAA0305, FGD1, Hrs-1, Hrs-2 and EEA1. Identical residues (dark grey) and conservative changes (light grey) are marked. A consensus sequence (bottom) was derived from positions in which at least 6 out of 7 residues were conserved or when proteins contained one of only two alternate residues.

The regulation of the subcellular localization of components of signaling pathways can be key determinants in the effective initiation and maintenance of signaling cascades. Targeting of signal transduction proteins to specific subcellular regions is highly regulated, often through specific interactions with scaffolding or anchoring proteins (Faux and Scott, 1996; Pawson and Scott, 1997). Scaffolding proteins have been defined as proteins that bind to multiple kinases to coordinate the assembly of a cascade, while anchoring proteins are tethered to specific subcellular regions in the cell and can act to bring together components of a pathway. Regulating location of signaling components can thus coordinate the activity of a signaling network, maintain signaling specificity or facilitate activation of a pathway by localizing kinases together with their downstream substrates.

As described herein, a recombinantly produced human SARA protein bound directly and specifically to unphosphorylated Smad2 and Smad3. In addition, receptor-dependent phosphorylation induced Smad2 to dissociate from SARA, bind to Smad4 and translocate to the nucleus. Thus, the hSARA1 protein functions in TGFβ signaling upstream of Smad activation to recruit Smad2 to the TGFβ receptor by mediating the specific subcellular localization of Smad and by associating with the TGFβ receptor complex. Furthermore, inducing mislocalization of Smad2 by expressing a mutant of the hSARA1 protein blocks TGFβ-dependent transcriptional responses, indicating an essential role for SARA-mediated localization of Smads in signaling. Together, these results identify the cloned hSARA1 protein as a novel component of the TGFβ pathway that

functions to anchor Smad2 to specific subcellular sites for activation by the TGFβ receptor kinase.

In vitro, receptor-regulated Smads are recognized by the receptor kinases and are phosphorylated on the C-terminal SSXS motif (Abdollah et al., 1997; Kretzschmar et al., 1997; Macias-Silva et al., 1996; Souchelnytskyi et al., 1997). This phosphorylation is similar to receptor-dependent phosphorylation in mammalian cells, suggesting that SARA is not absolutely required for recognition of Smads by the receptor complex. In intact cells, however, receptor-regulated Smads are cytosolic proteins that require activation by transmembrane serine/threonine kinase receptors. Consequently, Smads may require recruitment by SARA to interact with TGFβ superfamily receptors. Domains in which SARA is found correspond to regions where TGFβ receptors are also localised. TGFβ receptors display regionalized localization and hSARA1 recruits Smad2 to these domains. The identity of these intracellular domains is unclear. However, they contain receptors and recent evidence has shown that FYVE finger domains interact with membranes, so it is reasonable to suggest that these domains represent membrane vesicles. Thus, clustering of the TGFβ receptor, as previously described by Henis et al. (1994), may function to direct the receptor to hSARA1 and the Smad2 substrate. This activity may be most critical *in vivo*, where ser/thr kinase receptors are often found in low numbers and only a small proportion need to be activated for biological responses (Dyson and Gurdon, 1998). This activity is likely to be most critical *in vivo*, where ser/thr kinase receptors are often found in low numbers and only a small proportion need to be activated for biological responses (Dyson and Gurdon, 1998). This may impose on the pathway a stringent requirement for SARA to anchor Smads in these sites for receptor interaction.

The colocalization and association of hSARA1 with the TGFβ receptor defines a role for hSARA1 in recruiting Smad2 to the receptor kinase. Furthermore, deletion of the FYVE domain interferes with receptor binding, prevents the correct localization of hSARA1/Smad2 and blocks TGFβ signaling (see Example 8 below), suggesting that this is an important function in the

pathway. Interestingly, the binding of the hSARA1 protein identified in Example 2 to the receptor was enhanced upon Smad2 expression and, on its own, SARA may interact inefficiently with the receptor. However, within the hSARA1/Smad complex, Smad2 might help drive association with the receptor through its recognition of the catalytic region of the kinase domain. Consistent with this, cooperation requires a kinase deficient type I receptor which also traps the Smad2 substrate (Macías-Silva et al., 1996). Thus, Smad2 may bind to the catalytic pocket of the type I kinase domain while hSARA1, which is not a substrate of the kinase, may interact with regions outside of the domain.

The human SARA protein identified in Example 2 did not interact with any of the other Smads tested, indicating that it functions specifically in Smad2 and Smad3 pathways (see Example 3). However, Smad5 localization in 293 cells displayed a remarkably similar pattern to that of this SARA protein (Nishimura et al., 1998) and similar patterns were observed for endogenous Smad1 or 5 in the kidney epithelial cell line, IMCD-3. Thus, localization of BMP-regulated Smads (for example, Smad1, Smad5 and Smad8) may also be regulated by a specific SARA family member.

The genes for two other SARA family member proteins were also identified and cloned. One of these, identified in *Xenopus* and designated *XSARA2* (Tables 7 and 8), is related to *XSARA1*, while the other one, *hSARA2* (Tables 3 and 4), is a human clone, related to the *hSARA1* of Tables 1 and 2. This second human clone has been identified in EST clone KIAA0305. A comparison of the SBD from hSARA1 with a similar region from the KIAA0305 sequence indicated that the amino terminal half of the region of the SBD was highly divergent from the amino acid sequence encoded by KIAA0305. This suggests that the protein encoded by KIAA0305 may mediate binding with other as yet unidentified proteins, eg. other Smads. In contrast to the SBD, the FYVE domain of the KIAA0305 protein is more closely related to the hSARA1 FYVE domain (70% identity), suggesting that this protein may be an anchor for other Smad proteins that function either in the TGFβ pathway or in other signaling cascades, such as the BMP signal transduction pathway.

SARA is not limiting in Smad activation and TGFβ superfamily signaling

It was observed that elevating Smad2 levels can saturate hSARA1 and yield a diffuse distribution for Smad2. Thus, the level of the hSARA1 protein is a key determinant in controlling Smad2 localization. As a consequence, endogenous Smad2 may or may not display a hSARA1-like distribution, depending on the relative expression of the two proteins. Indeed, in Mv1Lu cells, endogenous Smad2 displays a punctate pattern with some diffuse staining in the cytosol. While not meaning to limit the invention to a particular mechanism, the data are consistent with the view that once signaling has commenced, Smad2 dissociates from hSARA1, binds to Smad4 and translocates to the nucleus, freeing hSARA1 to recruit additional Smad2 from the cytosolic reservoir. This would provide a mechanism to allow quantitative activation of Smads in the presence of high levels of TGFβ signaling.

By functioning to recruit Smad2 to the TGFβ receptor, hSARA1 is located in an important regulatory position in the pathway. Thus, control of hSARA1 localization or protein levels, or its interaction with Smad2, could modulate TGFβ signaling. Further, disruption of normal hSARA1 function could potentially be involved in loss of TGFβ responsiveness that is a common feature during tumour progression.

Modular Domains in SARA

The function of hSARA1 in TGFβ signaling is mediated by three independent domains, the Smad binding domain (SBD) that mediates specific interaction with Smad2 and Smad3, the FYVE domain that targets hSARA1/Smad2 to specific subcellular sites and the carboxy terminus which mediates association with the TGFβ receptor. The *Xenopus* and mouse forkhead-containing DNA binding proteins, FAST1 and FAST2, bind specifically to Smad2 and Smad3 and like hSARA1, interact with the MH2 domains (Chen et al., 1996; Chen et al., 1997a; Labbé et al., 1998; Liu et al., 1997a). Comparison of the SBD from this SARA with the Smad Interaction Domain (SID)

from these FAST proteins revealed no regions of obvious similarity. However, since hSARA1 acts upstream and FAST downstream of Smad activation, these proteins may employ structurally unrelated domains to distinguish unactivated versus activated forms of Smad2. Thus, the SBD of this SARA protein preferentially binds unphosphorylated monomeric Smad2 while the SID from FAST must bind phosphorylated Smad2 in heteromeric complexes with Smad4. By analogy, the SBD of other SARA family members may bind the unphosphorylated monomeric species of other Smads that mediate signal transduction in other pathways (eg. Smads 1, 5 or 8 in the BMP signal transduction pathway).

In hSARA1, the FYVE domain functions independently of the SBD, to mediate the subcellular targetting of the protein. The FYVE-finger motif has now been identified in at least 30 proteins from diverse species, such as FGD1, Hrs-1 and 2, and EEA1 (Gaullier et al., 1998; Wiedemann and Cockcroft, 1998). Recent advances have demonstrated that FYVE finger motifs from a variety of divergent proteins have a conserved function and bind phosphatidyl inositol-3-phosphate (PtdIns(3)P) with high specificity (Burd et al., (1998); Patki (1998); Gaullier (1998)). Through this interaction, the FYVE finger can mediate protein interactions with phospholipid bilayers. However, PtdIns(3)P is present ubiquitously on cell membranes and in the case of EEA1, further protein-protein interactions with Rab5-GTP are required in addition to the FYVE domain to target the protein to the correct membranes (Simonsen et al., 1998). Given that PtdIns(3)P binding by FYVE fingers is conserved in yeast and mammals, it is likely that the FYVE finger of hSARA1 similarly mediates interaction with the membrane. Furthermore, it is possible that additional protein-protein interactions may be required to direct hSARA1 to regions that contain the TGFβ receptors. The carboxy terminus of hSARA1, which is required for efficient interaction with the TGFβ receptor, may function in this capacity.

Together, these data define discrete domains in SARA that fulfill specific aspects of SARA function in TGFβ superfamily signaling. Without being limited to any particular mechanism, a possible model of the interaction of SARA with a

receptor regulated Smad in TGFβ superfamily signaling, as exemplified by hSARA1 and its interactions with Smad2 in TGFβ signaling, is shown diagrammatically in Figure 18. The FYVE domain likely functions to direct SARA to the membrane, perhaps through interactions with PtIns(3)P. It thus fulfills an important role in recruiting hSARA1 to specific subcellular domains that have been shown also to contain the TGFβ receptor. The SBD in turn functions to bind unactivated Smad2, thus recruiting the receptor substrate to this subcellular region. Once localized to this region, the C-terminal domain of hSARA1 functions with Smad2 bound to the SBD to promote interaction with the receptor complex. These three domains thus function cooperatively to recruit Smad2 to the TGFβ receptor.

Additional Roles for SARA

Controlling the localization of kinases and their substrates may allow not only for efficient recognition and phosphorylation but may also function to maintain specificity and suppress crosstalk between signaling pathways. Thus, by controlling Smad localization, a SARA family member protein could additionally function to maintain the highly specific regulation of Smad phosphorylation by ser/thr kinase receptors that is observed *in vivo* and could prevent promiscuous phosphorylation by other kinases in the cell. Furthermore, through its interactions with a particular receptor, a SARA protein might function to control the activity or turnover of the receptor complex. Alternatively, SARA may also fulfill scaffolding functions to coordinate the receptor-dependent activation of Smads with other as yet unidentified components of a signaling pathway.

Proteins

SARA proteins may be produced by culturing a host cell transformed with a DNA sequence encoding a selected SARA protein. The DNA sequence is operatively linked to an expression control sequence in a recombinant vector so that the protein may be expressed.

Host cells which may be transfected with the vectors of the invention may be selected from the group consisting of *E. coli*, *Pseudomonas*, *Bacillus subtillus*, or other bacilli, yeasts, fungi, insect cells or mammalian cells including human cells.

For transformation of a mammalian cell for expression of a SARA protein, the vector may be delivered to the cells by a suitable vehicle. Such vehicles including vaccinia virus, adenovirus, retrovirus, Herpes simplex virus and other vector systems known to those of skill in the art.

A SARA protein may also be recombinantly expressed as a fusion protein. For example, the SARA cDNA sequence is inserted into a vector which contains a nucleotide sequence encoding another peptide (e.g. GST-glutathione succinyl transferase). The fusion protein is expressed and recovered from prokaryotic (e.g. bacterial or baculovirus) or eukaryotic cells. The fusion protein can then be purified by affinity chromatography based upon the fusion vector sequence and the SARA protein obtained by enzymatic cleavage of the fusion protein.

The protein may also be produced by conventional chemical synthetic methods, as understood by those skilled in the art.

SARA proteins may also be isolated from cells or tissues, including mammalian cells or tissues, in which the protein is normally expressed.

The protein may be purified by conventional purification methods known to those in the art, such as chromatography methods, high performance liquid chromatography methods or precipitation.

For example, anti-SARA antibodies may be used to isolate SARA protein which is then purified by standard methods.

Antibodies

The provision of the polynucleotide and amino acid sequences of SARA proteins provides for the production of antibodies which bind selectively to a SARA protein or to fragments thereof. The term "antibodies" includes polyclonal antibodies, monoclonal antibodies, single chain antibodies and fragments thereof such as Fab fragments.

In order to prepare polyclonal antibodies, fusion proteins containing defined portions or all of a SARA protein can be synthesized in bacteria by expression of the corresponding DNA sequences, as described above. Fusion proteins are commonly used as a source of antigen for producing antibodies. Alternatively, the protein may be isolated and purified from the recombinant expression culture and used as source of antigen. Either the entire protein or fragments thereof can be used as a source of antigen to produce antibodies.

The purified protein is mixed with Freund's adjuvant and injected into rabbits or other appropriate laboratory animals. Following booster injections at weekly intervals, the animals are then bled and the serum isolated. The serum may be used directly or purified by various methods including affinity chromatography to give polyclonal antibodies.

Alternatively, synthetic peptides can be made corresponding to antigenic portions of a SARA protein and these may be used to inoculate the animals.

In a further embodiment, monoclonal anti-SARA antibodies may be produced by methods well known in the art. Briefly, the purified protein or fragment thereof is injected in Freund's adjuvant into mice over a suitable period of time, spleen cells are harvested and these are fused with a permanently growing myeloma partner and the resultant hybridomas are screened to identify cells producing the desired antibody. Suitable methods for antibody preparation may be found in standard texts such as Antibody Engineering, 2d. edition, Barreback, ED., Oxford University Press, (1995).

EXAMPLES

The examples are described for the purposes of illustration and are not intended to limit in any way the scope of the invention.

Methods of molecular genetics, protein and peptide biochemistry and immunology referred to but not explicitly described in this disclosure and examples are reported in the scientific literature and are well known to those skilled in the art.

Example 1: Methods

Isolation of *Xenopus* and human SARA

To prepare a probe for library screening, the MH2 domain of Smad2 (amino acids 241-467) was subcloned into a modified pGEX4T-1 vector containing the protein kinase A recognition site derived from pGEX2TK (Pharmacia). This bacterial fusion protein was purified, labelled with [^{32}P]γATP and used as probe to screen a λZAP II *Xenopus* dorsal lip library as described (Chen and Sudol, 1995). A screen of 1 x 10^6 plaques yielded four phage which represented repeated isolates of the same clone. This partial cDNA contained a 2.1 kb open reading frame and 1 kb of 3′ untranslated region (UTR). A full length clone was obtained by a combination of rescreening of the same dorsal lip library using a 670 base pair EcoRI/HpaI fragment at the 5′ end of this clone and by 5′ RACE (Gibco/BRL) using stage 10 Xenopus RNA.

To obtain a human homolog of *Xenopus* SARA, cDNA was synthesized from randomly primed total RNA isolated from HepG2 cells. This cDNA was subjected to polymerase chain reaction (PCR) using degenerate primers as described previously (Attisano et al., 1992). The 5′ and 3′ primers, designed to encode the zinc-finger motif, correspond to GC(A/C/G/T/)CC(A/C/G/T)AA(C/T)TG(C/TATGAA(A/C/G/T)TG(C/T) and (A/G)CA(A/G)TA(C/T)TC(A/C/G/T)GC(A/C/G/T)GG(A/G)TT(A/G)TT, respectively. A 150 base pair PCR product was sequenced and then used as probe for screening a λZAP human fetal brain cDNA library (Stratagene). Eight positive plaques were obtained, two of which contained an overlap of approximately 1kb

and covered the entire open reading frame. The sequence of the 5' UTR was confirmed by sequencing of an expressed sequence tag database clone (clone ID 260739).

Construction of Plasmids

For mammalian expression constructs of SARA, the open reading frame of hSARA was amplified by PCR and was subcloned into pCMV5 in frame with an amino-terminal Flag or Myc tag (Hoodless et al., 1996). The deletion mutants of pCMV5-Flag-hSaraΔ893-1323, Δ346-132, Δ893-1323, and Δ346-1323 were constructed by deletion of EcoRV-HindIII, XbaI-HindIII, SalI-EcoRV, and SalI-XbaI fragments, respectively. PCMV5-Flag-hSaraΔ1-594 and Δ1-686 were obtained by partial digestion with Asp718/SalI and for pCMV5-Flag-hSARA Δ665-1323 a Asp718/HindIII partial digest was used. PCMV5-Flag-hSARAΔ596-704 was constructed by deleting Asp718 fragment. The other hSARA mutants were constructed by PCR using appropriate primers. PCMV5B-Myc-Smad3 and Myc-Smad6, pGEX4T-1-Smad2/MH1 (amino acids 1-181), pGEX4T-1-Smad2/linker (amino acids 186-273), pGEX4T-1-Smad2/MH2 (amino acids 241-467) and pGEX4T-1-h SARA (amino acids 665-750) were constructed by PCR.

***In Vitro* Protein Interactions**

In vitro transcription/translation reactions were performed using the TNT coupled reticulocyte lysate system (Promega) following the manufacturer's instructions using T3 RNA polymerase. Translation was carried out in the presence of [^{35}S]-methionine and labelled proteins were incubated with purified GST fusion proteins in TNTE buffer with 10% glycerol for 2 hours at 4°C and then washed five times with the same buffer. Bound protein was separated by SDS-PAGE and visualized by autoradiography.

Immunoprecipitation and Immunoblotting

COS-1 cells transfected with LipofectAMINE (GIBCO BRL) were lysed with lysis buffer (Wrana et al., 1994) and subjected to immunoprecipitation with

either anti-Flag M2 (IBI, Eastern Kodak) or anti-Myc (9E10) monoclonal antibody followed by adsorption to protein-G sepharose. Precipitates were separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted as described previously (Hoodless et al., 1996).

Affinity-Labelling

LipofectAMINE transfected COS-1 cells were incubated with 200 pM [^{125}I]TGFβ in media containing 0.2% bovine fetal serum at 37°C for 30 minutes and receptors were cross-linked to ligand with DSS as described previously (Macias-Silva et al., 1996). Cell lysates were immunoprecipitated with anti-Flag antibody and receptors visualized by SDS-PAGE and autoradiography. In some cases, cross-linked [^{125}I]TGFβ was determined by gamma counting.

Subcellular Localization by Immunofluorescent Confocal Microscopy

Mv1Lu cells, plated on gelatin-coated Permanox chamber slides (Nunc), were transfected by the calcium phosphate-DNA precipitation method. Fixation, permeabilisation and reaction with the primary and secondary antibodies were described previously (Hoodless et al., 1996). Monoclonal anti-Flag antibodies were visualized by FITC-conjugated goat anti-mouse IgG (Jackson Laboratories) and polyclonal Myc antibody (A14, Santa Cruz) was visualized with Texas-Red-conjugated goat anti-rabbit IgG (Jackson Laboratories). Immunofluorescence was analyzed on a Leica confocal microscope.

Transcriptional Response Assay

Mv1Lu cells were transiently transfected with the reporter plasmid, p3TP-lux (Wrana et al., 1992), CMV-βgal and selected constructs using calcium phosphate transfection. Twenty-four hours after transfection, cells were incubated overnight with or without 50 pM TGFβ. Luciferase activity was measured using the luciferase assay system (Promega) in a Berthold Lumat LB 9501 luminometer and was normalized to β-galactosidase activity.

Example 2 - Identification of SARA family members

The MH2 domain of Smad2 was fused to glutathione-S-transferase (GST) that included a kinase recognition site for protein kinase A (PKA). The bacterially-expressed fusion protein was labelled to high specific activity using PKA (Chen and Sudol, 1995), and then used to screen a λZAPII expression library prepared from the dorsal blastopore lip of *Xenopus*. From this screen, four clones were identified, all of which presented a repeated isolate of a partial cDNA clone with no similarity to sequences in the GenBank database. To confirm that the product encoded by this clone interacted with Smad2, an *in vitro* transcription/translation system was used to produce [^{35}S]methionine-labelled protein. Translation of the cDNA yielded a protein product of approximately 80 kDa which corresponded in size to the longest open reading frame (ORF) identified in the sequence. Incubation of this product with bacterially-produced GST-Smad2(MH2) resulted in efficient binding of the translated product to the fusion protein (data not shown). Interaction with full length Smad2 was also observed, whereas binding to bacterially-expressed Smad1 or Smad4 was not.

To isolate a full length cDNA, the partial clone identified in the interaction screen was used as a probe to rescreen the same blastopore lip library. Since the resulting clones lacked the 5' end, 5' RACE was conducted to obtain the entire coding sequence. Analysis of the complete cDNA sequence (Table 5) revealed a long open reading frame that was contiguous with that of the partial clone. The predicted protein, XSARA1, is 1235 amino acids long with an estimated molecular mass of 135 kDa (Table 6). Analysis of the full length cDNA sequence (Table 9) revealed a region in the middle portion of the predicted protein that had similarity to a double zinc finger domain (recently renamed the FYVE domain; Mu et al., 1995). The FYVE domain has been identified in a number of unrelated signaling molecules that include FGD1, a putative guanine exchange factor for Rho/Rac that is mutated in faciogenital dysplasia (Pasteris et al., 1994), the HGF receptor substrate Hrs-1 and its homolog Hrs-2 (Bean et al., 1997; Komada and Kitamura, 1995), EEA1, a protein

involved in formation of the early endosome (Mu et al., 1995) and the yeast proteins FAB1, VPS27 and VAC1 (Piper et al., 1995; Weisman and Wickner, 1992; Yamamoto et al., 1995). Comparison of the FYVE domains from the vertebrate proteins with that from SARA revealed extensive conservation of residues throughout the domain (Table 10). Thus, SARA contains a FYVE domain that may fulfill important functions in diverse proteins.

To investigate the role of SARA in TGFβ superfamily signaling in mammalian cells, a human homologue was identified. Using a carboxy-terminal portion of XSARA1, a human library was screened and a protein was identified that was distantly related to *Xenopus* SARA (34% identity) and which was also sequenced as an EST (KIAA0305). However, no homologs closer to XSARA were identified. Thus, degenerate oligonucleotide primers were designed encoding amino acids in XSARA1 (Table 9) and HepG2 RNA was used as template for degenerate PCR. A related sequence was identified and this partial cDNA was used to screen a human brain cDNA library. Four overlapping clones, encoding a long open reading frame were identified and a search of the EST database with this sequence led to the identification of additional overlapping cDNA clones from libraries derived from T cells, uterus, endothelial cells and melanocytes. Analysis of the contiguous sequence revealed a long open reading frame that had a consensus start codon preceded by stop codons in all three reading frames (Table 1). Comparison of the predicted protein hSARA1 (Table 2), from this cDNA with XSARA1 (Table 9) revealed an overall identity of 62%, with a divergent 558 residue amino terminal domain (35% identity) followed by a closely related carboxy terminus (85% identity).

Example 3 - hSARA interacts specifically with Smad2 and Smad3

To characterize the interaction of hSARA with Smads, the full length protein was translated *in vitro* and tested for binding to bacterially-expressed Smad fusion proteins. Similar to the *Xenopus* clone, hSARA1 bound specifically to full length Smad2, but not Smad1 or Smad4 (Figure 1). In addition, full length Smad3, which is highly related to Smad2, also interacted with hSARA1. To

define the domains of Smad2 that bound hSARA, in bacteria various fragments of Smad2 corresponding to the MH1 domain, linker region and MH2 domain were expressed in bacteria. Similar to the *Xenopus* clone, hSARA interacted efficiently with fusion proteins that comprised the MH2 domain, while no association was detected between hSARA and either the MH1 or non-conserved linker domains (Figure 1). Thus, hSARA1 specifically interacts with Smad2 through the MH2 domain.

To confirm that hSARA also bound to Smads in mammalian cells, a Flag epitope tag was introduced at the amino terminus of the protein to create Flag-SARA. Transient expression of Flag- SARA in COS cells yielded a protein of the predicted molecular weight for SARA (Figure 2) that was not present in untransfected cells (data not shown). To investigate the interaction of SARA with Smads, Flag- SARA was expressed in COS cells together with Myc-tagged versions of Smads 1, 2, 3, 4, 6 and 7. Cell lysates were subjected to anti-Flag immunoprecipitation followed by immunoblotting with anti-Myc antibodies. In other immunoprecipitates of cells expressing either Smad2 or Smad3, efficient coprecipitation of either Smad with Flag- hSARA1 was observed (Figure 2). In contrast, none of the other Smads coprecipitated with hSARA1. Specific binding of this SARA family member to both Smad2 and Smad3 is consistent with the observation that these two proteins possess very closely related MH2 domains (97% identity) and are both activated by TGFβ or activin type I receptors (Liu et a., 1997b; Macias-Silva et al., 1996; Nakao et al., 1997a). Together, these results demonstrate that this SARA family member is a specific partner for receptor-regulated Smads of the TGFβ/activin signaling pathway.

Example 4- Phosphorylation of Smad2 induces dissociation from SARA

Previous findings have shown that activation of TGFβ signaling results in phosphorylation of Smad2 or Smad3 by type I receptors on C-terminal serine residues (Liu et al., 1997b; Macias-Silva et al., 1996). A constitutively active TGFβ type I receptor was prepared by substituting a threonine in the GS domain with an aspartate residue (Wieser et al., 1995). This activated type I receptor

induces TGFβ signaling in the absence of type II receptors and ligand and regulates the phosphorylation and activation of Smad proteins in a manner similar to ligand (Macias-Silva et al., 1996; Wieser et al., 1995). COS cells were transfected with combinations of Smad2, hSARA1 or both in the presence or absence of activated TβRI. Cells were then metabolically labelled with [^{32}P]phosphate and phosphorylation of either hSARA1 or Smad2 was assessed in immunoprecipitates. Analysis of SARA phosphorylation revealed that the protein was basally phosphorylated and the coexpression of the activated type I receptor did not appreciably affect the overall phosphorylation (Figure 3). In contrast, analysis of Smad2 immunoprecipitated from total cell lysates showed that the activated type I receptor induced strong phosphorylation of the protein as described previously (Macias-Silva et al., 1996). These results suggest that SARA is not phosphorylated in response to TGFβ signaling.

The phosphorylation state of Smad2 that coprecipitated with hSARA1 was examined. Interestingly, unlike the strong induction of Smad2 phosphorylation in the total cellular pool, phosphorylation of Smad2 associated with hSARA1 was not enhanced, but rather appeared to decrease in the presence of TGFβ signaling (Figure 3). This suggested that receptor-dependent phosphorylation of Smad2 might induce dissociation from hSARA1. To examine this directly, the interaction of hSARA1 with wild type Smad 2 or a mutant version lacking the C-terminal phosphorylation sites (Smad2(2SA)) was analysed. In the absence of TGFβ signaling, association of hSARA1 with either Smad2 or Smad2(2SA) was comparable (Figure 4). In contrast, in cells coexpressing the activated receptor, a significant decrease in the interaction of wild type Smad2 with hSARA1 was observed. However, hSARA1/Smad2(2SA) complexes were not reduced by the activated receptor. Together, these results suggest that hSARA1 is not phosphorylated in response to TGFβ signaling and that it preferentially interacts with the unphosphorylated form of Smad2.

Example 5 - SARA and Smad4 form mutually exclusive complexes with Smad2

Phosphorylation of Smad2 induces its interaction with Smad4 (Lagna et al., 1996; Zhang et al., 1997). hSARA1/Smad2 complexes in COS cells coexpressing Smad4 were assessed. In unstimulated cells, the level of hSARA1/Smad2 complex formation was comparable either in the presence or absence of Smad4 (Figure 5, lanes 3 and 6). However, upon activation of TGFβ signaling, dissociation of Smad2 from hSARA1 was significantly enhanced by coexpression of Smad4 (Figure 5, lanes 4 and 7). These results indicated that phosphorylated Smad2 might preferentially interact with Smad4 rather than hSARA1 and suggested that Smad2 might form mutually exclusive complexes with either Smad4 or hSARA1. The formation of Smad2/Smad4 and Smad2/hSARA4 complexes in the same transfectants was then examined. Cell lysates were subjected to immunoprecipitation with anti-Flag antibodies directed towards tagged Smad2 and then immunoblotted for the presence of Smad4 and hSARA1. Consistent with previous findings (Lagna et al., 1996; Zhang et al., 1997), interaction of Smad4 with Smad2 was strongly stimulated by the activated type I receptor (Figure 6, lane 3 and 4). Concomitant with the formation of Smad2/Smad4 complexes, the interaction of Smad2 with hSARA1 was disrupted by activation of signaling (Figure 6, lanes 6 and 7). Thus, complexes of Smad2/hSARA1 and Smad2/Smad4 are mutually exclusive, supporting the notion that Smad4 may compete for Smad2 to enhance dissociation of hSARA1/Smad2 complexes. Together these results demonstrate that during TGFβ signaling, hSARA1/Smad2 complexes are transient and phosphorylation of Smad2 induces dissociation and formation of heteromeric complexes with Smad4.

Example 6- hSARA1 regulates the subcellular localization of Smad2

The studies described above suggest that SARA functions upstream in the pathway and might control the subcellular localization of Smad2. To test this, an investigation was done to determine whether coexpression of hSARA1 might alter the localization of Smad2 in the TGFβ-responsive epithelial cell line, Mv1Lu, using confocal microscopy. Mv1Lu cells were used rather than COS

since the Myc antibodies crossreacted with endogenous proteins in the COS and obscured nuclear staining of tagged proteins. In cells expressing hSARA1 alone, the protein displayed a punctate staining pattern that was present throughout the cytosolic compartment and was excluded from the nucleus (Figure 7A). This localization of hSARA1 was in contrast to the diffuse staining typically observed for Smad2 in cells overexpressing the protein (Figure 7B). Cells transiently transfected with both hSARA1 and Smad2 were examined. In these cells, the distribution of hSARA1 was indistinguishable from cells transfected with hSARA1 alone (Figure 7D, left photo). In contrast, the localization of Smad2 in the presence of hSARA1 displayed a dramatic shift to a punctate pattern (compare Figure 7B to 7D, centre photos). Moreover, analysis of these immunofluorescent staining patterns by confocal microscopy revealed that hSARA1 and Smad2 precisely colocalized in the cytosol (yellow stain, Figure 7D, right photo). Interestingly, expression of Smad2 at much higher levels than hSARA1 reverted the distribution of Smad2 to that observed in cells transfected with Smad2 alone (data not shown). This supports the notion that elevating the amount of Smad2 can saturate hSARA1 and yield a diffuse distribution of Smad2 throughout the cell.

Studies were conducted to determine whether activation of TGFβ signaling induces nuclear translocation of Smad2 in the presence of hSARA1. As shown in Figure 7, the localization of hSARA1 in the cytosolic compartment looked similar in the presence or absence of the constitutively active TGFβ type I receptor (compare Figure 7D and E, left photos). However, TGFβ signaling caused a significant proportion of Smad2 to translocate to the nucleus (Figure 7E, centre photo) and this correlated with a shift to an orangy-red colour in the cytosolic colocalization stain (Figure 7E, right photo). Thus activation of TGFβ signaling induces Smad2 to dissociate from hSARA1 and translocate to the nucleus.

To confirm that the punctate localization of overexpressed SARA reflected that of the endogenous protein, the localization of endogenous SARA and Smad2 was examined in Mv1Lu cells. Analysis of the distribution of endogenous

hSARA1 using affinity-purified rabbit anti-hSARA1 antibodies revealed a punctate distribution that was similar to the pattern observed for transiently transfected, epitope-tagged hSARA1 (Figure 7F, left photo). This staining was specific, since cells stained with preimmune antisera, or purified antibody blocked with the hSARA1 antigen, revealed no detectable staining in the cytosol, although some weak background staining was observed in the nucleus (data not shown). Examination of endogenous Smad2 distribution in the same cell using goat anti-Smad2 antibodies revealed a punctate distribution for Smad2 (Figure 7F, centre photo) as published previously (Janknecht et al., 1998). Furthermore, analysis of hSARA1 and Smad2 together revealed extensive colocalization of the two proteins (Figure 7F, right photo). Colocalization was not complete and may reflect differences in the stoichiometry of hSARA1 versus Smad2 protein levels as suggested above, or the presence of additional regulatory mechanisms in the cell that control interaction of the endogenous proteins.

Taken together with the biochemical analysis, these results indicate that hSARA1 functions to anchor or recruit Smad2 to specific subcellular regions prior to activation by TGFβ signaling.

Example 7 - hSARA1 co-localises with TβRII

The positioning of hSARA1 upstream of Smad2 activation suggested to us that hSARA1 might recruit Smad2 to specific subcellular domains for phosphorylation and activation by the receptor. Interestingly, previous studies on the TGFβ receptor demonstrated clustering of the receptor complex into punctate domains that resembled those displayed by hSARA1 (Henis et al., 1994). To test whether hSARA1 might colocalize with TGFβ receptors, the subcellular localization of hSARA1 and TGFβ Mv1Lu receptors was investigated in Mv1Lu cells. Endogenous TGFβ receptors could not be detected, likely due to the low numbers of TGFβ receptors present on these cells and the even fewer number that are activated in the presence of ligand. The localization of hSARA1 in Mv1Lu cells cotransfected with TβRII and treated with TGFβ was therefore examined. In the absence of hSARA1, TβRII displayed a punctate staining

pattern similar to the hSARA1 pattern (Figure 8A, panels i and ii, respectively), as observed previously in COS cells. Furthermore, in cells coexpressing hSARA1 and TGFβ receptors, extensive colocalization of hSARA1 and TβRII was observed (Figure 8A, panel iii). This colocalization was not complete. This may be due to a restricted distribution of hSARA1 in only a subset of the intracellular compartments normally occupied by transmembrane receptors, which include the endoplasmic reticulum, Golgi and endocytic pathways. Thus, hSARA1 and the TGFβ receptors colocalize to common subcellular domains.

The colocalization of hSARA1 and the TGFβ receptors suggested the possiblity that hSARA1 may interact with the TGFβ receptor. To test this, a strategy was utilised similar to that employed to characterize the interaction of Smad2 with the TGFβ receptor (Macías-Silva et al., 1996). Briefly, COS cells were cotransfected with TGFβ receptors in the presence of hSARA1 and were affinity-labelled using [^{125}I]TGFβ. hSARA1 was then immunoprecipitated from the cell lysates and coprecipitating receptor complexes were resolved by SDS-PAGE and visualized by autoradiography or were quantitated using a gamma counter. Analysis of cells expressing wild type receptors type II and type I, revealed that receptor complexes coprecipitated with hSARA1 (Figure 8B, lane 3). Furthermore, in the presence of kinase deficient type I receptor, there was a small increase in binding of hSARA1 to the receptor (Figure 8B, lane 2). This is in contrast to Smad2, which only interacts with TGFβ receptor complexes that contain kinase deficient type I receptors (Macías-Silva et al., 1996). These data suggest that hSARA1 associates with the TGFβ receptor.

Next examined was whether coexpression of Smad2 might enhance the interaction of hSARA1 with TGFβ receptors. In cells expressing wild type receptor I, no difference in the amount of receptor complexes that coprecipitated with hSARA1, either in the presence or absence of Smad2, was observed (Figure 8B, compare lanes 3 and 5). In contrast, the association of hSARA1 with receptor complexes containing kinase-deficient type I receptors was enhanced by Smad2 (Figure 8B, lane 4). This finding was consistent with the previous demonstration that kinase-deficient type I receptors stabilize interactions of

Smad2 with the receptors. To investigate further the requirement for Smad2 in the interaction of hSARA1 with the receptor, a mutant of hSARA1, SARA(ΔSBD), that removes the Smad binding domain, was tested. Analysis of wild type hSARA1 interaction with receptor complexes containing kinase-deficient TβRI showed that wild type hSARA1 interacted with the receptor and this was enhanced approximately two-fold by Smad2 (Figure 9A). The ΔSBD mutant of hSARA1 retained the capacity to associate with the receptor, although the efficiency of interaction was slightly reduced relative to wild type hSARA1. Importantly, unlike wild type hSARA1, binding of mutant hSARA1 to the receptor was not enhanced by coexpression of Smad2. Together, these data suggest that hSARA1 interacts with the TGFβ receptor independently of Smad2 binding and that Smad2 cooperates to enhance the association.

To further characterize the domains in SARA that mediate binding to the TGFβ receptor, the interaction of a panel of SARA mutants with the TGFβ receptor was tested. Interestingly, interaction with the TGFβ receptor was strongly suppressed in three mutants in which the FYVE domain was disrupted (Figure 9B; Δ594, Δ664 and the internal deletion Δ597-665). Since the FYVE domain is required for the correct subcellular localization of SARA, it was postulated that, once bound to the membrane, other regions in SARA might contribute to the interaction with the receptor. To examine this possibility, several carboxy-terminal truncation mutants of hSARA1 were tested. Interestingly, deletion of the C-terminus downstream of position 750 suppressed receptor interaction, despite efficient expression of the truncated protein. This suggests that regions in the carboxy-terminus of SARA contribute to receptor interaction. In these analyses, the question of whether overexpression of Smad2 could rescue some interaction of SARA mutants with the receptor was also explored. For both the FYVE domain mutants and the C-terminal truncation, Smad2 expression was able to restore some interaction with the TGFβ receptor. It is likely that the high levels of protein and receptor expression that are achieved in COS cells can drive some receptor interaction, even in the absence of appropriate localization signals.

Example 8 - A modular domain in SARA mediates association with Smads

To investigate the functional importance of SARA in TGFβ signaling, the domains in the protein that mediate both its localization to specific subcellular regions and its interaction with Smad2 were defined. To this end, a series of deletion mutants of hSARA1 were constructed and tested for their ability to interact with Smad2 in COS cells by immunoprecipitation followed by immunoblotting. As summarized in Figure 10, loss of the first 665 amino acids of hSARA1, which included the double zinc finger/FYVE domain, did not interfere with hSARA1 binding to Smad2. However, further deletions (Δ1-704) completely abolished the interaction of Smad2 with hSARA1. To map the carboxy-terminal boundary of the Smad binding domain, a number of C-terminal truncations were also analyzed. Deletion of all residues downstream of position 750 did not affect Smad2 interaction with hSARA1, while an additional loss of 85 amino acids (Δ665-1323) completely abrogated binding to Smad2. To determine whether the region defined by this deletional analysis was sufficient to bind Smad2, the 85 amino acids referred to as the Smad Binding Domain (SBD) were linked to GST and the fusion protein was expressed in bacteria (GST-h SARA(665-750)). Incubation of lysates prepared from cells expressing Smad2 or Smad3 with GST-SBD resulted in efficient binding of both Smads to the fusion protein (Figure 11A). This interaction is likely direct, since bacterially expressed SBD associates efficiently with bacterially-produced Smad2 (data not shown). These studies thus define a novel domain in SARA that mediates interaction with Smad2 and Smad3 and which is located downstream of the FYVE domain.

The above-described analysis in COS cells showed that phosphorylation of Smad2 by the TGFβ receptor induced dissociation from SARA. To determine whether this reflects an alteration in the ability of the SBD to bind phosphorylated Smad2, the interaction of GST-SBD with Smad2 in lysates obtained from cells expressing Smad2 alone, or Smad2 together with either wild type or activated TGFβ type I receptor, was tested. As described previously, coexpression of activated type I receptors with the appropriate receptor-

regulated Smad yields efficient phosphorylation of Smad protein. In lysates from cells expressing Smad2 alone or Smad2 with wild type receptors, efficient binding of Smad2 to GST-SBD was observed. In contrast, in the presence of activated TβRI, the interaction of Smad2 with GST-SBD was strongly reduced (Figure 11B). This reduction correlated with receptor-dependent phosphorylation, since the phosphorylation site mutant, Smad2(2SA), interacted efficiently with GST-SBD, even in the presence of activated TβRI (data not shown). These data strongly support a mechanism whereby SARA interacts with unphosphorylated Smad2 and receptor-dependent phosphorylation induces dissociation by altering the affinity of Smad2 for the SBD.

Example 9 - The FYVE domain controls the subcellular localization of SARA

The subcellular localization of a selection of the SARA mutants was analysed by immunofluorescence and confocal microscopy. Analysis of truncation mutants that removed the amino terminus upstream of the FYVE domain (Δ1-531) yielded wild type patterns of staining (Figure 12, compare panels i and ii). However, a further deletion (Δ1-664) that disrupted the FYVE domain but did not interfere with the Smad binding domain, abolished the wild type staining pattern (Figure 12, panel iii). Similar studies of the C-terminal domains showed that residues downstream of the FYVE domain (Δ665-1323) did not alter the localization of the mutant protein (Figure 12, panel iv), while truncations within the FYVE domain (Δ596-1323) led to diffuse localization throughout the cell (Figure 12, panel v). Of note, the Δ665-1323 mutant lacked the Smad binding domain, thereby indicating that interaction with Smad2 is not required for proper SARA localization. To confirm that FYVE domain function was required for localization of SARA, a mutant with a small internal deletion that removes the FYVE domain (Δ597-664) was tested. Consistent with the other mutants, localization of this protein was clearly disrupted (Figure 12, panel vi). Since none of these mutants interfered with Smad binding, the FYVE domain appears to be required to maintain the normal localization of SARA but is not involved in mediating interactions with Smads.

Example 10 - SARA-mediated localization of Smad2 is necessary for TGFβ signaling

The availability of mutants of hSARA1 that interact with Smad2 but fail to target to the appropriate subcellular sites allowed the question of whether hSARA1-mediated localization of Smad2 was important to TGFβ signaling to be addressed. Whether SARA(Δ1-594) and SARA(Δ1-664), which bind Smad but fail to distribute to the correct subcellular domains, would mislocalize Smad2 was examined. Coexpression of either mutant with Smad2 showed that they were unable to recruit Smad2 to the normal SARA domains (Figure 13A, panels i and ii). As expected, SARA(Δ1-704), which lacks a Smad binding domain, was unable to control Smad2 localization (Figure 13A, panel iii). Whether these mutants could cause mislocalization of Smad2 was also examined. For this, cells were cotransfected with wild type hSARA1 and Smad2 either in the absence or presence of SARA(Δ1-594), SARA(Δ1-664) or SARA(Δ1-704). In control transfectants, performed in the absence of mutant hSARA1, hSARA1 and Smad2 were colocalized in punctate domains as described above (Figure 13B, panel i). However, in the presence of either SARA(Δ1-594) or SARA(Δ1-664), the localization of wild type hSARA1 was normal, but the distribution of Smad2 was clearly disrupted and displayed a diffuse pattern (Figure 13B, panels ii and iii, respectively). Moreover, coexpression of SARA(Δ1-704), which does not bind Smad2, resulted in Smad2 distribution that was indistinguishable from that of the wild type pattern (Figure 13B, panel iv). Thus, SARA(Δ1-594) and SARA(Δ1-664) induce the mislocalization of Smad2.

Since SARA(Δ1-664) mislocalizes Smads and interferes with receptor association, we investigated whether this mutant would disrupt TGFβ signaling. To test this, we transiently transfected the TGFβ-responsive reporter gene 3TP-lux into Mv1Lu cells in the presence and absence of wild type or mutant versions of hSARA1. Expression of wild type hSARA1 had no effect on TGFβ signaling (Figure 14). In contrast, transfection of SARA(Δ1-664) significantly inhibited TGFβ-dependent signaling at the lowest concentration of DNA tested,

while transfection of higher doses completely abolished responsiveness of the cells. We also tested SARA(Δ1-704) which lacks a functional Smad binding domain and does not alter Smad2 localization. Transfection of this mutant had no effect on TGFβ signaling (Figure 14). In addition to analysis of the 3TP promoter, we examined induction of the activin response element (ARE) from the Xenopus Mix.2 gene in HepG2 cells.

This ARE is stimulated by either TGFβ or activin signaling, which induces assembly of a DNA binding complex that is composed of Smad2, Smad4 and a member of the FAST family of forkhead DNA binding proteins. Since HepG2 cells do not possess endogenous FAST activity, wild type or mutants of hSARA1 were cotransfected with FAST2 and the ARE-lux reporter plasmid as described previously (Labbé et al., 1998). Expression of either SARA(1-Δ594) or SARA(1-Δ664), which interfere with or delete the FYVE domain, respectively, resulted in a strong suppression of TGFβ-dependent induction of the ARE (Figure 15). However, none of the other mutants tested suppressed activation of this promoter. Since none of these latter mutants disturb the localization of hSARA1-Smad2 complexes, these data strongly suggest that recruitment of Smad2 to the receptor-containing subcellular domains is important for TGFβ signaling.

Example 11 - Tissue distribution of hSARA expression

The 3'UTR of hSARA1 and a Smad2 cDNA fragment were used to probe a human multiple tissue Northern blot (Clontech). The results are shown in Figure 16 - hSARA1: upper panel and Smad2: lower panel. hSARA1 and Smad2 were ubiquitously expressed in the tissues examined; relatively low levels of hSARA1 were selected in liver. hSARA1 and Smad2 showed a similar expression pattern except in placenta, where proportionally more Smad2 message was observed. A single transcript of 5.0 kb is seen, corresponding to the full length hSARA1 cDNA.

SARA expression was examined in a variety of cell lines using RT-PCR analysis and the gene was found to be expressed in every cell line tested. These included HepG2 hepatoma cells, NBFL neuroblastoma cells, SW480 colorectal

cancer cells, N1H 3T3 fibroblasts, P19 embryonic carcinoma cells, MC3T3 calvarial cells and Mv1Lu lung epithelial cells (data not shown). hSARA1 appears to be a ubiquitously expressed partner for Smad2 and Smad3.

Example 12 - Interaction of endogenous hSARA1 and Smad2 in mammalian cells

Lysates from HepG2 cells, either untreated or treated with InM TGFβ, were immunoprecipitated with an affinity-purified, anti-hSARA1 rabbit polyclonal antibody and the immunoprecipitates were immunoblotted with a polyclonal, anti-Smad2 antibody (Macias-Silva et al., 1998). Controls were immunoprecipitated with pre-immune sera or N19 anti-Smad2/3 antibody. The results are shown in Figure 17. In immunoprecipitates prepared with preimmune antisera, no Smad2 was detectable. Anti-hSARA1 immunoprecipitates clearly showed Smad2 co-precipitating with hSARA1. TGFβ treatment prior to lysis gave decreased association of Smad2 and SARA.

These results demonstrate that SARA is a specific partner of receptor-regulated Smads in the TGFβ/activin signaling pathway and further suggest that TGFβ signaling induces dissociation of SARA/Smad complexes.

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TABLE 1 - hSARA1

GCATACTGAATCAGCAGGACTGGCTGGTGGTGCAGCAGACATCATGAGTAAGCACCG
AGAAGTCTGTTCCTTATCACGTGTGTAAGGGGAAAAAGGTTTAAACAAGTCTCTTAA
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GCAGCAAATTATCTATCTAATGGTTGTGATTCCTATGGAATGCAAGACCCAGGTGTT
TCTTTTGTTCCAAAGACTTTACCCTCCAAAGAAGATTCAGTAACAGAAGAAAAAGAA
ATAGAGGAAAGCAAGTCAGAATGCTACTCAAATATTTATGAACAGAGAGGAAATGAG
GCCACAGAAGGGAGTGGACTACTTTTAAACAGCACTGGTGACCTAATGAAGAAAAAT
TATTTACATAATTTCTGTAGTCAAGTTCCATCAGTGCTTGGGCAATCTTCCCCCAAG
GTAGTAGCAAGCCTGCCATCTATCAGTGTTCCTTTTGGTGGTGCAAGACCCAAGCAA
CCTTCTAATCTTAAACTTCAAATTCCAAAGCCATTATCAGACCATTTACAAAATGAC
TTTCCTGCAAACAGTGGAAATAATACTAAAAATAAAAATGATATTCTTGGGAAAGCA
AAATTAGGGGAAAACTCAGCAACCAATGTATGCAGTCCATCTTTGGGAAACATCTCT
AATGTCGATACAAATGGGGAACATTTAGAAAGTTATGAGGCTGAGATCTCCACTAGA
CCATGCCTTGCATTAGCTCCAGATAGCCCAGATAATGATCTCAGAGCTGGTCAGTTT
GGAATTTCTGCCAGAAAGCCATTCACCACGCTGGGTGAGGTGGCTCCAGTATGGGTA
CCGGATTCTCAGGCTCCAAATTGCATGAAATGTGAAGCCAGGTTTACATTCACCAAA
AGGAGGCATCACTGCAGAGCATGTGGGAAGGTTTTCTGTGCTTCCTGCTGTAGCCTG
AAATGTAAACTGTTATACATGGACAGAAAGGAAGCTAGAGTGTGTGTAATCTGCCAT
TCAGTGCTAATGAATGCTCAAGCCTGGGAGAACATGATGAGTGCCTCAAGCCAGAGC
CCTAACCCTAACAATCCTGCTGAATACTGTTCTACTATCCCTCCCTTGCAGCAAGCT
CAGGCCTCAGGAGCTCTGAGCTCTCCACCTCCCACTGTGATGGTACCTGTGGGAGTT
TTAAAGCACCCTGGAGCAGAAGTGGCTCAGCCCAGAGAGCAGAGGCGAGTTTGGTTT
GCTGATGGGATCTTGCCCAATGGAGAAGTTGCTGATGCAGCCAAATTAACAATGAAT
GGAACTTCCTCTGCAGGAACCCTGGCTGTGTCACACGACCCAGTCAAGCCAGTAACT
ACCAGTCCTCTACCAGCAGAGACGGATATTTGTCTATTCTCTGGGAGTATAACTCAG
GTTGGAAGTCCTGTTGGAAGTGCAATGAATCTTATTCCTGAAGATGGCCTTCCTCCC
ATTCTCATCTCCACTGGTGTAAAAGGAGACTATGCTGTGGAAGAGAAACCATCACAG
ATTTCAGTAATGCAGCAGTTGGAGGATGGTGGCCCTGACCCACTTGTATTTGTTTTA
AATGCAAATTTGTTGTCAATGGTTAAAATTGTAAATTATGTGAACAGGAAGTGCTGG
TGTTTCACAACCAAGGGAATGCATGCAGTGGGTCAGTCTGAGATAGTCATTCTTCTA

TABLE 1 - hSARA1 Continued

CAGTGTTTACCGGATGAAAAGTGTTTGCCAAAGGATATCTTTAATCACTTTGTGCAG
CTTTATCGGGATGCTCTGGCAGGGAATGTGGTGAGCAACTTGGGACATTCCTTCTTC
AGTCAAAGTTTCCTTGGCAGTAAAGAACATGGTGGATTCTTATATGTGACATCTACC
TACCAGTCACTGCAAGACCTAGTACTCCCAACCCCACCTTACTTGTTTGGGATTCTT
ATCCAGAAATGGGAAACTCCTTGGGCTAAAGTATTTCCTATCCGTCTGATGTTGAGA
CTTGGAGCTGAATATCGACTTTATCCATGCCCACTATTCAGTGTCAGATTTCGGAAG
CCATTGTTTGGAGAGACGGGGCATACCATCATGAATCTTCTTGCAGACTTCAGAAAT
TACCAGTATACCTTGCCAGTAGTTCAAGGTTTGGTGGTTGATATGGAAGTTCGGAAA
ACTAGCATCAAAATTCCCAGCAACAGATACAATGAGATGATGAAAGCCATGAACAAG
TCCAATGAGCATGTCCTGGCAGGAGGTGCCTGCTTCAATGAAAAGGCAGACTCTCAT
CTTGTGTGTGTACAGAATGATGATGGAAACTATCAGACCCAGGCTATCAGTATTCAC
AATCAGCCCAGAAAAGTGACTGGTGCCAGTTTCTTTGTGTTCAGTGGCGCTCTGAAA
TCCTCTTCTGGATACCTTGCCAAGTCCAGTATTGTGGAAGATGGTGTTATGGTCCAG
ATTACTGCAGAGAACATGGATTCCTTGAGGCAGGCACTGCGAGAGATGAAGGACTTC
ACCATCACCTGTGGGAAGGCGGACGCGGAGGAACCCCAGGAGCACATCCACATCCAG
TGGGTGGATGATGACAAGAACGTTAGCAAGGGTGTCGTAAGTCCTATAGATGGGAAG
TCCATGGAGACTATAACAAATGTGAAGATATTCCATGGATCAGAATATAAAGCAAAT
GGAAAAGTAATCAGATGGACAGAGGTGTTTTTCCTAGAAAACGATGACCAGCACAAT
TGCCTCAGTGATCCTGCAGATCACAGTAGATTGACTGAGCATGTTGCCAAAGCTTTT
TGCCTTGCTCTCTGTCCTCACCTGAAACTTCTGAAGGAAGATGGAATGACCAAACTG
GGACTACGTGTGACACTTGACTCAGATCAGGTTGGCTATCAAGCAGGGAGCAATGGC
CAGCCCCTTCCCTCGCAGTACATGAATGATCTGGATAGCGCCTTGGTGCCGGTGATC
CATGGAGGGGCCTGCCAGCTTAGTGAGGGCCCCGTTGTCATGGAACTCATCTTTTAT
ATTCTGGAAAACATCGTATAAACAGAGAAGACTTCATTTTTTTCTGTTCAGACTTGT
TGCAACAGCAGTCATACCCAAATCATTTGCACTTTAAAACTGGAAGATTAAGCTTTT
GTTAACACTATTAATGGGGTGGGGAATAGGGTGGGAGTGGGGGTTTGGGAGACGGGT
GGGAAAGGGTGGTTGGGGGGACCGATGTTCCATAATTCTAAGTCTTCTATGCATTGT
CCACCAAGAAGATCTGGGCAGCTTCTGTTCCTGCACAACAGTTATGCTATCCTTGCA
GCTAATCCCCTTCTGTTACTGTTTAGACAAGAATTCCGCTCCTCTCTCAAGATTTAC
TTATGGTCATGTGCTCAGAAATGCTCAAATGGGTACAACCATCACCAAGGGTGGGAT
GGGAGGGCAGAGGGGAAATAAAATATAAAGCATCAAAAAAAAAAAAAAAAA

TABLE 2 - hSARA1

MWIDENAVAEDQLIKRNYSWDDQCSAVEVGEKKCGNLACLPDEKNVLVVAVMHNCDK
RTLQNDLQDCNNYNSQSLMDAFSCSLDNENRQTDQFSFSINESTEKDMNSEKQMDPL
NRPKTEGRSVNHLCPTSSDSLASVCSPSQLKDDGSIGRDPSMSAITSLTVDSVISSQ
GTDGCPAVKKQENYIPDEDLTGKISSPRTDLGSPNSFSHMSEGILMKKEPAEESTTE
ESLRSGLPLLLKPDMPNGSGRNNDCERCSDCLVPNEVRADENEGYEHEETLGTTEFL
NMTEHFSESQDMTNWKLTKLNEMNDSQVNEEKEKFLQISQPEDTNGDSGGQCVGLAD
AGLDLKGTCISESEECDFSTVIDTPAANYLSNGCDSYGMQDPGVSFVPKTLPSKEDS
VTEEKEIEESKSECYSNIYEQRGNEATEGSGLLLNSTGDLMKKNYLHNFCSQVPSVL
GQSSPKVVASLPSISVPFGGARPKQPSNLKLQIPKPLSDHLQNDFPANSGNNTKNKN
DILGKAKLGENSATNVCSPSLGNISNVDTNGEHLESYEAEISTRPCLALAPDSPDND
LRAGQFGISARKPFTTLGEVAPVWVPDSQAPNCMKCEARFTFTKRRHHCRACGKVFC
ASCCSLKCKLLYMDRKEARVCVICHSVLMNAQAWENMMSASSQSPNPNNPAEYCSTI
PPLQQAQASGALSSPPPTVMVPVGVLKHPGAEVAQPREQRRVWFADGILPNGEVADA
AKLTMNGTSSAGTLAVSHDPVKPVTTSPLPAETDICLFSGSITQVGSPVGSAMNLIP
EDGLPPILISTGVKGDYAVEEKPSQISVMQQLEDGGPDPLVFVLNANLLSMVKIVNY
VNRKCWCFTTKGMHAVGQSEIVILLQCLPDEKCLPKDIFNHFVQLYRDALAGNVVSN
LGHSFFSQSFLGSKEHGGFLYVTSTYQSLQDLVLPTPPYLFGILIQKWETPWAKVFP
IRLMLRLGAEYRLYPCPLFSVRFRKPLFGETGHTIMNLLADFRNYQYTLPVVQGLVV
DMEVRKTSIKIPSNRYNEMMKAMNKSNEHVLAGGACFNEKADSHLVCVQNDDGNYQT
QAISIHNQPRKVTGASFFVFSGALKSSSGYLAKSSIVEDGVMVQITAENMDSLRQAL
REMKDFTITCGKADAEEPQEHIHIQWVDDDKNVSKGVVSPIDGKSMETITNVKIFHG
SEYKANGKVIRWTEVFFLENDDQHNCLSDPADHSRLTEHVAKAFCLALCTQLKLLKG
DGMTKLGLRVTLDSDQVGYQAGSNGQHLPSQYMNDFDSDLVKMIHGGACQLSEGPVV
MELIFYILENIV

TABLE 3 - human SARA2

ACTCCCGGCCGGGGTAGCTCTTCACTCCTCAGCGCGACGTCGTGTCGAGTTCCCAAA
AAGCTCCGCAGGGGCTGTAGGGAGGTGATCTCATCCATTAACAGCTGTGTGTTGCCA
GTTCCCAAATCTTTATCTATCTCAGACTTCTCTCCTGCATTCCAGATTCTTATATTC
AGCTGCCTTTTGGATATCTCTCCCAGGATGTTCTCAAGGCATACAAGAATTAAATTC
TGAATAAGTCTGCAGGTAGGATGGACAGTTATTTTAAAGCAGCTGTCAGTGACTTGG
ACAAACTCCTTGATGATTTTGAACAGAACCCAGATGAACAAGATTATCTCGCAGATG
TACAAAATGCATATGATTCTAACCACTGCTCAGTTTCTTCAGAGTTGGCTTCCTCAC
AGCGAACTTCATTGCTCCCAAAAGACCAAGAGTGCGTTAATAGTTGTGCCTCATCAG
AAACAAGCTATGGAACAAATGAGAGTTCCCTGAATGAAAAAACACTCAAGGGACTTA
CTTCTATACAAAATGAAAAAAATGTAACAGGACTTGATCTTCTTTCTTCTGTGGATG
GTGGTACTTCAGATGAAATCCAGCCGTTATATATGGGACGATGTAGTAAACCTATCT
GTGATCTGATAAGTGACATGGGTAACTTAGTTCATGCAACCAATAGTGAAGAAGATA
TTAAAAAATTATTGCCAGATGATTTTAAGTCTAATGCAGATTCCTTGATTGGATTGG
ATTTATCTTCAGTGTCAGATACTCCCTGTGTTTCTTCAACAGACCATGATAGTGATA
CTGTCAGAGAACAACAGAATGATATCAGTTCTGAATTACAAAATAGAGAAATCGGAG
GAATCAAAGAATTGGGTATAAAAGTAGATACAACACTTTCAGATTCCTATAATTACA
GTGGAACAGAAAATTTAAAAGATAAAAAGATCTTTAATCAGTTAGAATCAATTGTTG
ATTTTAACATGTCATCTGCTTTGACTCGACAAAGTTCCAAAATGTTTCATGCCAAAG
ACAAGCTACAACACAAGAGCCAGCCATGTGGATTACTAAAAGATGTTGGCTTAGTAA
AAGAGGAAGTAGATGTGGCAGTCATAACTGCCGCAGAATGTTTAAAAGAAGAGGGCA
AGACAAGTGCTTTGACCTGCAGCCTTCCGAAAAATGAAGATTTATGCTTAAATGATT
CAAATTCAAGAGATGAAAATTTCAAATTACCTGACTTTTCCTTTCAGGAAGATAAGA
CTGTTATAAAACAATCTGCACAAGAAGACTCAAAAAGTTTAGACCTTAAGGATAATG
ATGTAATCCAAGATTCCTCTTCAGCTTTACATGTTTCCAGTAAAGATGTGCCGTCCT
CATTGTCCTGTCTTCCTGCGTCTGGGTCTATGTGTGGATCATTAATTGAAAGTAAAG
CACGGGGTGATTTTTTACCTCAGCATGAACATAAAGATAATATACAAGATGCAGTGA
CTATACATGAAGAAATACAGAACAGTGTTGTTCTAGGTGGGGAACCATTCAAAGAGA
ATGATCTTTTGAAACAGGAAAAATGTAAAAGCATACTCCTTCAGTCATTAATTGAAG
GGATGGAAGACAGAAAGATAGATCCTGACCAGACAGTAATCAGAGCTGAGTCTTTGG
ATGGTGGTGACACCAGTTCTACAGTTGTAGAATCTCAAGAGGGGCTTTCTGGCACTC
ATGTCCCAGAGTCTTCTGATTGTTGTGAAGGTTTTATTAATACTTTTTCAAGCAATG
ATATGGATGGGCAAGACTTAGATTACTTTAATATTGATGAAGGCGCAAAAAGTGGCC
CACTAATTAGTGATGCTGAACTTGATGCCTTTCTGACAGAACAGTATCTTCAGACCA
CTAACATAAAGTCTTTTGAAGAAAATGTAAATGACTCTAAATCGCAAATGAATCAGA
TAGATATGAAAGGCTTAGATGATGGAAACATCAATAATATATATTTCAATGCAGAAG
CAGGAGCTATTGGGGAAAGTCATGGTATTAATATAATTTGTGAAACAGTTGATAAAC
AAAATACAATAGAAAATGGCCTTTCTTTAGGAGAAAAAAGCACTATTCCAGTTCAAC
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CAAGGAGTTCAAAGGACCTGAATAAGCCAGATGTTCCAGATACAATAGAAAGTGAAC
CCAGCACAGCAGATACCGTTGTTCCAATCACTTGTGCTATAGATTCTACAGCTGATC
CACAGGTTAGCTTCAACTCTAATTACATTGATATAGAAAGTAATTCTGAAGGTGGAT
CTAGTTTCGTAACTGCAAATGAAGATTCTGTACCTGAAAACACTTGCAAAGAAGGCT
TGGTTTTGGGCCAGAAACAGCCTACTTGGGTTCCTGATTCAGAAGCTCCAAACTGTA
TGAACTGCCAAGTCAAATTTACTTTTACCAAACGGCGACACCATTGCCGAGCATGTG
GGAAAGTATTTTGTGGTGTCTGTTGTAATAGGAAGTGTAAACTGCAATATCTAGAAA
AGGAAGCAAGAGTATGTGTAGTCTGCTATGAAACTATTAGTAAAGCTCAGGCATTTG
AAAGGATGATGAGTCCAACTGGTTCTAATCTTAAGTCTAATCATTCTGATGAATGTA
CTACTGTCCAGCCTCCTCAGGAGAACCAAACATCCAGTATACCTTCACCAGCAACTT
TGCCAGTCTCAGCACTTAAACAACCAGGTGTTGAAGGACTATGTTCCAAAGAACAGA
AGAGAGTATGGTTTGCAGATGGTATATTGCCCAATGGTGAAGTTGCAGATACAACAA
AATTATCATCTGGAAGTAAAAGATGTTCTGAAGACTTTAGTCCTCTCTCACCTGATG
TGCCTATGACAGTAAACACAGTGGATCATTCCCATTCTACTACAGTGGAAAAGCCAA
ACAATGAGACAGGAGATATTACAAGAAATGAGATAATTCAGAGTCCTATTTCTCAGG

TABLE 3 - human SARA2 Continued

TTCCATCAGTGGAAAAATTGTCTATGAACACAGGAAATGAGGGGTTACCTACTTCTG
GTTCATTTACACTAGATGATGATGTTTTTGCAGAAACTGAAGAACCATCTAGTCCTA
CTGGTGTCTTAGTTAACAGCAATTTACCTATTGCTAGTATTTCAGATTATAGGTTAC
TGTGTGATATTAACAAGTATGTCTGCAATAAGATTAGTCTTCTACCTAATGATGAGG
ACAGTTTGCCCCCACTTCTGGTTGCATCTGGAGAAAAGGGATCAGTGCCTGTAGTAG
AAGAACATCCATCTCATGAGCAGATCATTTTGCTTCTTGAAGGTGAAGGCTTTCATC
CTGTTACATTTGTCCTAAATGCTAATCTACTCGTGAATGTCAAATTCATATTTTATT
CCTCAGACAAATATTGGTACTTTTCAACCAATGGATTGCATGGCTTGGGACAGGCAG
AAATTATTATTCTATTGTTATGTTTGCCAAATGAAGATACTATTCCTAAGGACATCT
TCAGACTATTTATCACCATATATAAGGATGCTCTAAAAGGAAAATACATAGAAAACT
TGGACAATATTACCTTTACTGAGAGTTTTCTCAGTAGCAAGGATCACGGAGGATTCC
TGTTTATTACACCTACTTTTCAGAAACTTGATGATCTCTCATTACCAAGTAATCCTT
TTCTTTGTGGAATTCTTATCCAGAAGCTTGAGATTCCCTGGGCAAAGGTTTTTCCTA
TGCGTTTAATGTTGAGATTGGGTGCAGAATATAAAGCATATCCTGCTCCTCTAACAA
GCATCAGAGGCCGAAAACCTCTTTTTGGAGAAATAGGACACACTATTATGAACTTAC
TTGTTGACCTTCGAAATTACCAGTATACCTTGCATAATATAGATCAACTGTTGATTC
ATATGGAAATGGGAAAAAGCTGCATAAAAATACCACGGAAAAAGTACAGTGATGTAA
TGAAAGTACTAAATTCTTCCAATGAGCATGTCATTAGCATTGGAGCAAGTTTCAGTA
CAGAAGCAGATTCTCATCTAGTCTGTATACAGAATGATGGAATTTATGAAACACAGG
CCAACAGTGCCACTGGCCATCCTAGAAAAGTGACAGGTGCAAGTTTTGTGGTATTCA
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GCTTAATGGTACAAATAACTCCAGAGACCATGAATGGCTTGCGGCTAGCTTTACGAG
AACAGAAAGACTTTAAAATTACATGTGGGAAAGTTGATGCAGTAGACCTGAGAGAAT
ACGTGGATATCTGCTGGGTAGATGCTGAAGAAAAAGGAAACAAAGGAGTTATCAGTT
CAGTGGATGGAATATCATTACAAGGATTTCCAAGTGAAAAAATAAAACTGGAAGCAG
ATTTTGAAACCGATGAGAAGATTGTAAAATGTACCGAGGTGTTCTACTTTCTAAAGG
ACCAGGATTTATCTATTTTATCAACTTCTTATCAGTTTGCAAAAGAAATAGCCATGG
CTTGTAGTGCTGCGCTGTGCCCTCACCTGAAAACTCTAAAAAGTAATGGGATGAATA
AAATTGGACTCAGAGTTTCCATTGACACTGATATGGTTGAATTTCAGGCAGGATCTG
AAGGCCAACTTCTGCCTCAGCATTATCTAAATGATCTTGATAGTGCTCTGATACCTG
TGATCCATGGTGGGACCTCCAACTCTAGTTTACCATTAGAAATAGAATTAGTGTTTT
TCATTATAGAACATCTTTTTTTAGTGAAAGAATGTGCCATATTACATATTGCAACCTA
ATTTGTTAAAACTAACTCCAGCACTAAAGCTGAAATGCCACAAACACTAAAAGTATA
AATATGTCTGATTTTTGAAACACATAAGCTTTGCTCTTTAGGCAGGAATGATCTTTT
CAAATCATTAGCACAATATTTAAATATCTAAAAATTTAAGAGATCCATACTTTCTGT
AGCTTTACAATTAATTTAAGTACTAAAAAGACAAGGATTTCTTTTAAGAAATTTATA
GCATTTACTGTGTTATTTAAATGCTAAGCCAAAGTATCTGCACTTAGGTATACCTCT
TTATGCCAATAATGATTTTAATGAAGGCTCTTTTCAGATGTAACCTTATGAAGGAAA
TATCTGCTTTGTGTATATGCCAGTTAGAATACTGGTTTCTAAAGTCTGTCAAATTGT
ATTTCAGTGGCACAAAAACCAGTTTTGAGGTCTTAGACTTATAATTCTTTGAATAAA
ACTGATAACTTATTTGTATAATTGGAGTGGAGACCTACCTCCATAATTAGATAAACT
CTTTTTGGATTATAATCAGAATTTTGCCTTTTTTCTTCTCAAATTATTACATATGTA
TGTATTATATATCCACATATATAGTTTTCCCTGATTAAATGGATATTAAAATAATTG
CGGGTGCTTCAGGACTTTTTGCTTCTATATTTAAGTATATTGTTTTTATAGCAAGAA
CATATTCTGAATGTTTTATAAATCTTTAATAATTTATATGTAGGTAATATTTTTGTA
TCACAATGCATTATTTTTTTTCCTCCTTTCCTTCCAAACTATACCACTGTATTTACC
ACTTCTAAGAGTGACTGACGACGGGCCAGATGACCCTTGAAGTAGTCATTATGTAGC
AATAAATGAAGCCTGAAACAGGTTTTTTTACTTCCACTTTAATCCTTAGAAATTTCT
TGGCAACTTCGCATATTTTCATTGACACTGGTGTATAAGTATAAATTTAAATGAACT
AATTACTTTTGCATATTTTAAATTCTTTATATGGTAGTTATTTTTTATAACAGGATA
TTAACATAAGTTAAATCCTATGTATTTGAAATTGTTACAGAGCTTTCCTCTTTACTT
CAAACAGCAAAAAAGTGGGGGGCATATTGTAGTCCTGTCATTTAAGTTATGTAAAAA
ATTTAATCATTATTTTGATGCTTTAAACATTCTCATGTGTAATATATGTTTTTGTAT
CAAAAACACTCATATATTTCAAGAAAAAGAAATTATGTTAAATAGCCCTGTTTTAAG

TABLE 3 - human SARA2 Continued

AAAAATATTTATGAAGCATCTCAACTTGAAGATCAAGTCAAAGTTATAACTCAGGAT
CTGAGGTCTCAAGCTAGGAGAGACTGAGAATTTTAATCAGTTTGGGCATATAGTTTG
GACTGAATCACATCTGTAGTACTTAGCCAAAGACAATTTGGAGGAGAATATCAGCCT
TCTGGAAGTAGCTACTTCCTGAACAATGTAAAGTGTCGCAGATATTCAATAAAATGG
CAACCTGTTATAATTTGTGAAATTTATTGAAATGGTGTAAGATGAAAACAATTGCAT
ATCAAACCCAATTTATGTTTTCTAAATATAGTGTATGTATTCTGCCATGTAAGTAAT
TGAACAGTCTTAAAATAACCAAATGGTAGAGGGCTGTTCCATGATGGGACAGCTTTG
GATTTGTTTTCATAAAATCTCTACATTCAATAAAAATTGGAATTATGTGCCTGAAGT
TTGGAGGCACATTTTGAAGT

TABLE 4 - human SARA2

MDSYFKAAVSDLDKLLDDFEQNPDEQDYLQDVQNAYDSNHCSVSSELASSQRTSLLPK
DQECVNSCASSETSYGTNESSLNEKTLKGLTSIQNEKNVTGLDLLSSVDGGTSDEIQP
LYMGRCSKPICDLISDMGNLVHATNSEEDIKKLLPDDFKSNADSLIGLDLSSVSDTPC
VSSTDHDSDTVREQQNDTSSELQNREIGGIKELGIKVDTTLSDSYNYSGTENLKDKKI
FNQLESIVDFNMSSALTRQSSKMFHAKDKLQHKSQPCGLLKDVGLVKEEVDVAVITAA
ECLKEEGKTSALTCSLPKNEDLCLNDSNSRDENFKLPDFSFQEDKTVIKQSAQEDSKS
LDLKDNDVIQDSSSALHVSSKDVPSSLSCLPASGSMCGSLIESKARGDFLPQHEHKDN
IQDAVTIHEEIQNSVVLGGEPFKENDLLKQEKCKSILLQSLIEGMEDRKIDPDQTVIR
AESLDGGDTSSTVVESQEGLSGTHVPESSDCCEGFINTFSSNDMDGQDLDYFNIDEGA
KSGPLISDAELDAFLTEQYLQTTNIKSFEENVNDSKSQMNQIDMKGLDDGNINNIYFN
AEAGAIGESHGINIICETVDKQNTIENGLSLGEKSTIPVQQGLPTSKSEITNQLSVSD
INSQSVGGARPKQLFSLPSRTRSSKDLNKPDVPDTIESEPSTADTVVPITCAIDSTAD
PQVSFNSNYIDIESNSEGGSSFVTANEDSVPENTCKEGLVLGQKQPTWVPDSEAPNCM
NCQVKFTFTKRRHHCRACGKVFCGVCCNRKCKLQYLEKEARVCVVCYETISKAQAFER
MMSPTGSNLKSNHSDECTTVQPPQENQTSSIPSPATLPVSALKQPGVEGLCSKEQKRV
WFADGILPNGEVADTTKLSSGSKRCSEDFSPLSPDVPMTVNTVDHSHSTTVEKPNNET
GDITRNEIIQSPISQVPSVEKLSMNTGNEGLPTSGSFTLDDDVFAETEEPSSPTGVLV
NSNLPIASISDYRLLCDINKYVCNKISLLPNDEDSLPPLLVASGEKGSVPVVEEHPSH
EQIILLLLEGEGFHPVTFVLNANLLVNVKFIFYSSDKYWYFSTNGLHGLGQAEIIILLL
CLPNEDTIPKDIFRLFITIYKDALKGKYIENLDNITFTESFLSSKDHGGFLFITPTFQ
KLDDLSLPSNPFLCGILIQKLEIPWAKVFPMRLMLRLGAEYKAYPAPLTSIRGRKPLF
GEIGHTIMNLLVDLRNYQYTLHNIDQLLIHMEMGKSCIKIPRKKYSDVMKVLNSSNEH
VISIGASFSTEADSHLVCIQNDGIYETQANSATGHPRKVTGASFVVFNGALKTSSGFL
AKSSIVEDGLMVQITPETMNGLRLALREQKDFKITCGKVDAVDLREYVDICWVDAEEK
GNKGVISSVDGISLQGFPSEKIKLEADFETDEKIVKCTEVFYFLKDQDLSILSTSYQF
AKEIAMACSAALCPHLKTLKSNGMNKIGLRVSIDTDMVEFQAGSEGQLLPQHYLNDLD
SALIPVIHGGTSNSSLPLEIELVFFIIEHLF

TABLE 5 - XSARA1

CTGTAAGTTTGACTATGTAGGAAAGCATTTCTGTTATCTATGAAGTATGTTTTAGAGT
CAGACCAATAACTAAACGGTTTTCTTTTTTTTGTTTATTTCCCCTCAGATGAGACTGT
CTCTCCAAAGCTATTAGATGCTAAGTGGAATCAAATCTTAGAACCGCATTCACATAAA
GTCGCTGATAACTCCGCCCTTGACAATGTCTGTAAATCAATCATTGCTATTGAAGCTC
ATCTCAAAGTCAGGTCACCCGGCTTGTCAGCCCTTGTGAGGTCCACATATGTGAATGG
AGAAGTAGGTATTGTGGCACCTGAAATGCCCAAAATGGTGATAGGAGACACCATTATG
GCAGAGGATTCACTTTTTAACAACACTGGTCCCTCTGAAATTGTATGCAACCCATCTA
CTGTGGAGAGTCAAAGTTTACAAGCTTTAGATGATCAATCAGTGAATATTCACAATGA
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AACTTTGACTGCTTGACCATGGATAACGAAATGCCTTTGCACAGTCAAATGAGTGTTG
ATGACAATGACAAAGAAACTGTAACAATTTCAGTCCTTCCAACAATCATACAGGATAC
TAGTAACGTAAGCACAGACCCAGCTATCAATAAACCTGGCACTAAAGAACCCCATAGA
GCATTAAAGGAAACCACATCAGTTATTCTGCCTGAAATAAAGCCTTACTCCACATGTG
CTGCCCTTTCGTTTGAAAATAACAATAAGGTTCCCAGTTATCAATTAAATAATACAGA
TCTACTCAGCGTTTCACCAGTGGTTGAAGCATGTAGTGAGCAGCAGCAAAAACATACA
TCTTCCTTGCATGAAGAAAAACTTTTTGAAGGTGTTTCTGCAACGGAGTCCTTTGCAG
CCACTGCTGCGGAAACTGTACTGGATAATGAGGCTCTCCGTAGTGCTGAATTCTTTGA
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TGTGGCCTCTCTCAAGAAAGCAATGAAAAGTTTTGTGCAAGTAAAGAGTTTGAAGGAG
GGGTAGATGCTAATGTCTTGTTGGAAAATGCATGTGTAGCTTATAAAGAAGCAATAGA
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AGGGGACCTGGCAGCTTGTGCCTTGCAGTGTCTCCAGACAGCCCAGACAACGATCTGC
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GTTGCAGTCTAAAATGCAAACTACAGTACATGGATAAAAAGGAGGCTCGTGTGTGTGT
TATTTGTCATTCTGTGCTTATGAATGCTCAAGCATGGGAGAACATGTTAAGTGCATCG
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AGCAGGCACAAGCTTCAGGAGCACTGAGTTCCCCACCTCCCACTGTCATGGTGCCAGT
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TGGTTTGCTGATGGAATATTACCCAACGGAGAGACTGCTGACTCAGATAATGCAAACG
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AACCTGGGGCATTCCTTCCTCAGCCAGAGTTTCCTGGGTAGTAAGGATCATGGTGGAT
TTCTTTATGTTGCACCAACCTACCAGTCCCTCCAGGACCTGGTTCTTCCTGCAGAGCC
GTACTTGTTTGGAATCCTTATTCAAAAGTGGGAGACTCCATGGGCCAAAGTGTTCCCC
ATTCGGCTTATGCTGCGTTTAGGTGCAGAATACAGATTGTACCCATGTCCACTCTTCA
GTGTTCGATACAGAAAACCTCTGTTTGGGGAAACCGGACACACCATCATTAATGTTCT
AGCCGATTTCAGAAACTATCAGTATACTCTGCCAGTGGTGCAGGGCTTGGTGGTGGAT
ATGGAAGTCAGAAAAACTAGCATTAAAATCCCCAGCAATAGATACAATGAGATGATGA
AAGCAATGAACAAATCCAATGAGCATGTGTTGGCCATAGGAGCATGCTTCAACCAGAT

TABLE 5 - XSARA1 Continued

GGCAGACTCTCACCTTGTGTGTGTGCAAAACGATGATGGCAATTACCAGACCCAGGCA
ATTAGTATCCACAAACAACCACGTAAAGTGACCGGGGCCAGCTTCTTTGTCTTCAGTG
GTGCACTAAAGTCTTCTTCCGGATACCTGGCCAAATCCAGCATAGTAGAAGATGGGGT
AATGGTTCAGATCACCGCAGAGAGCATGGATGCCCTCAGACAGTCCCTTCGGGAGATG
AAGGATTTCACCATTACATGTGGAAAAGCTGATGCAGAGGAGTCACAGGAACATGTCT
ATGTCCAGTGGGTGGAGGATGACAAGAACTTTAACAAAGGAGTTTTTAGTCCAATCGA
TGGCAAATCAATGGAGTCTGTGACCAGCGTCAAGATTTTTCATGGCTCAGAATACAAA
GCTAGTGGAAAAATAATTCGCTGGATAGAGGTCTTCTTTCTGGACAATGAGGAGCAAC
AGAGTGGCCTGAGTGACCCTGCTGATCACAGCCGACTCACTGAAAATGTGGCCAAAGC
ATTCTGTTTAGCGCTTTGCCCACACCTCAAGCTACTGAAGGAAGATGGAATGACCAGG
TTAGGTCTGCGGGTGTCACTGGACTCAGACCAGGTTGGATACCAAGCTGGGAGCAATG
GGCAACTCCTGCCTGCCCGATACACCAATGATTTGGATGGTGCTTTGGTACCAGTGAT
ACACGGGGGCACATGCCAGTTAAGTGAAGGGCCTGTCAGTATGGAGCTGATATTTTAT
ATCCTTGAGAACATCTCCTAGGAAAGACACATGTGTCTCCTCACAAACTGCCATCGCC
CAAACCATTTGCACTTTAACCGCAAAAGATTCATTTTTCTTTTCTTTTGCTAACACTA
GTATTAGGTCAGGGTGCGAGAGGCAGACACCTGAACTCTTAAACCTTCTATGCATTTT
CACAGTAAGGATCAAGCTGCAGCTGGGAATTTCCTGTTACTAATCCAATGTGGGACGT
TAGAAGTGATCGGTGGCACTGACTATCTAGCTGTTCAACCTTCTCTGGCTCCTCTAAG
GACTCTAGTGCCAGGGGGTGAGACATTCAAGTTTAAAACGAAAACTCTAAATACAATC
AGGAATCTCACTCTGACCTCATTTAAATCATCACTGCGACTTTTTTTCCTGCTCGCAT
TCTTTATTTTGCATCTTACTCAAGTTTACATTGTCAAGACCAGCCTAAGCCTTCAGTC
CTTTCTCAATTAAACTACTCGTGCATGGCAAGGAGACTTTCGTTGCACAGCCTGAAAT
ATACCAATCACTTCCCAAACCACAAGCATGAATCCAACGTTTTCCTGACTGGTTGGCT
CTGCTGTGAAAGGGACAGCAATATTATTTTTCTACAGTTGACAAAACTTTTGTCTATG
TCTGTGTCTCTCATGGGGGATTTGTTGCCTGATGGGCAGCCTCCGGAGAGAAGAATTC
CACCCGTGTGTAATATACAGTCTAAGTGTATGGTCTGCTATGTAACACCTGTTGCGCA
GTGCAAATGCACTGACTCTCTGGAAGGCTATAGAGTTTTAAAAACGGTTAGTCTTTTA
AAAAAAAAA

TABLE 6 - XSARA1

MPKMVIGDTIMAEDSLFNNTGPSEIVCNPSTVESQSLQALDDQSVNIHNEKSVLLADG
FSPCSSPKSIINFDCLTMDNEMPLHSQMSVDDNDKETVTISVLPTIIQDTSNVSTDPA
INKPGTKEPHRALKETTSVILPEIKPYSTCAALSFENNNKVPSYQLNNTDLLSVSPVV
EACSEQQQKHTSSLHEEKLFEGVSATESFAATAAETVLDNEALRSAEFFDIVVKNFSD
SCVINGDLTKSCGLSQESNEKFCASKEFEGGVDANVLLENACVAYKEAIDLPEENGTN
APMSLYNGCDSYGMKNPAVAQNPKNLPSKEDSVTEEKEIEESKSEYYTGVYEQQREDD
VTERGGLLLNAKADQMKNNLHSLCNQVPSMHGQTSPKKGKIVQSLSVPYGGARTKQPT
HLKLHIPKPLSEMLQSDLIPPNAGCSSKYKNDMLNKSNQGDNLISESLREDSAVRSPV
TDANGDFPGEYRGPGSLCLAVSPDSPDNDLLAGQFGVPISKPFTTLGEVAPVWVPDSQ
APNCMKCEARFTFTKRRHHCRACGKVFCAACCSLKCKLQYMDKKEARVCVICHSVLMN
AQAWENMLSASVQSPNPNNPAEYCSTIPPMQQAQASGALSSPPPTVMVPVGVLKHPGT
EGSQSKEQRRVWFADGILPNGETADSDNANVTTVAGTLTVSHTNNSTSSESENTSGFC
GSITQVGSAMNLIPEDGLPPILISTGVKGDYAVEERPSQMSVMQQLEEGGPDPLVFVL
NANLLAMVKIVNYVNRKCWCFTTKGMHAVGQAEIVILLQCLPDEKCLPRDLFSHFVEL
YQEAIAGNVVGNLGHSFLSQSFLGSKDHGGFLYVAPTYQSLQDLVLPAEPYLFGILIQ
KWETPWAKVFPIRLMLRLGAEYRLYPCPLFSVRYRKPLFGETGHTIINVLADFRNYQY
TLPVVQGLVVDMEVRKTSIKIPSNRYNEMMKAMNKSNEHVLAIGACFNQMADSHLVCV
QNDDGNYQTQAISIHKQPRKVTGASFFVFSGALKSSSGYLAKSSIVEDGVMVQITAES
MDALRQSLREMKDFTITCGKADAEESQEHVYVQWVEDDKNFNKGVFSPIDGKSMESVT
SVKIFHGSEYKASGKIIRWIEVFFLDNEEQQSGLSDPADHSRLTENVAKAFCLALCPH
LKLLKEDGMTRLGLRVSLDSDQVGYQAGSNGQLLPARYTNDLDGALVPVIHGGTCQLS
EGPVSMELIFYILENIS*

TABLE 7 - XSARA2

AGTTTTATTTTCAGAAGACGTTGCATCTTTATTTTAAACATTAAGTTTCACTATGTAG
TAAAACATTACTGTTGTATATACAGTATGTTGTAGACATATAACGTAACTGTTTGCTT
TGTGCTTTCTTTCCTCCTCAGATGAAACTGTCTTTCCAAAGCTGTTAGATGCTAAGTG
GAATCAATTCTTAGAACCACATTCGCATAAAGTCACTGATAAACCAGCTCTTGACAAT
GTCTGTAAATCAATCATTGCTATTGAAGCTCATCTCAAAGTCAGGTCACCCAGCTTGA
CAGCCCTTGCAAGGTCCACATATGTGAATGGAGAAGTAGGTATTGTGACTCCTGAAAT
GCCTAAAATGGTGATAGGAGACACCGATATGGCAGAGGATTCACTTTTTAACACTGGT
CCCTCTGAAATTGTATGCAACTCTATTGTGGAGAGTCAAAGTTTAGAAGTTTTAGATG
ATGTACCAGTGAGTATTAACAATGAAAAAAGTGTTCTTCTTGATGATGGATTTTCTCC
GTACAGTAGCCCCAAAAGTGTTCTAAACTCTGCTTGCTTGACCATGAATAACGGAAAG
CCCTCACACGGTCAAAAAATTGTTAATGACCAAGATAAAGAAGCTGTAACAATTTCAG
TCCTTCCAATGATCATACAGGATACTACTAACGTAAGCACAGACCCAGCTTTCAATAA
ATCTGGCACTGAAGAAGCTTATAGTGCATTAAAACAAACCACATCAGTTATTCTGCCT
GAAATAAAGCCTTATTCCATACAGGCTGCCCTTTCATGTGAAAATATCAACAAGATAC
CCAGATGTCAATTAAATAATACAGATCTACTCAGCATTTCACCAGTGGTTGAAGCATG
TAGTGAGAAGCAGCAAAATCATACAACTTCCTTGCATGAAAAAAAACTTGCAGCTGTG
TCTGCAACTGCGTTCTTTCCAGTCACTGCTGCTGAAACTGTACTAGGTAATGAAGCTC
TCCATAGTGCTGATTTTTTTGACATTGTTGTAAAGAACGTTTCTGACTCGTGTGTGTT
TAATGGTGACCTAACTAGAACTAATGGACTCTCACAAGAAAACAATGAAATGTTTTAT
GCAAGTAAAGAGTTGGAAGGAGGGGTAGATGCTAATATCTTATTGGAAGATGCATGCA
TAGCTTATAAAGAAAGAATAGATTTGTCTGAAGAAAATGGAACTAATGCACCAATGTA
TCTGTACAATGGGTGTGATTCCTATGGAATGAAAAACCCTGCTGTACGTCAAAACCCA
AAGAATTTACCATCAAAAGAAGATTCTGTGACAGAAGAAAAAGAAATTGAAGAAAGCA
AGTCAGAATACTATTCTGGTGTTTATGAACAACAGAAGGAAGATGACATAACTGAGAG
AGGTGGAGTCTTGTTAAATGCCAAGGTTGACCAAATGAAGAACAGTTTGCATAGTCTT
TATAATCCGGTTCCATCCATGCATGGGCAAACCTCACCAAAAAAGGGCAAGATTGTGC
AATCCCTCAGTGTTCCATATGGTGGAGCTCGCCCCAAGCAGCCAACTCATCTCAAACT
CAATATTCCACAGCCATTGTCTGAAATGTTACAGTGTGATCTCATTCCGCCAAATGCT
GGATGCAGCTCTAAAAACAAAAATGACATGTTAAACAAATCAAATCGGGGGGATAACC
TGATTTCAGAATCACTACGTGAGGAAGTGCACAGCCCTGTTACTGATACAAATGGTGA
AGTCCCTCGAGAAAACAGGGGACCTGGCAGCCTGTGCCTTGCAGTGTCTCCAGACAGC
CCTGACAATGATCTGCTTGCTGGACAGTTTGGGGTACCCATCTCTAAGCCATTTACTA
CTCTAGGGGATGTGGCTCCAGTCTGGGTGCCAGATTCCCAAGCACCAAACTGCATGAA
GTGCGAGGCCAGATTTACATTTACCAAAAGGAGGCATCACTGCCGAGCTTGTGGAAAG
GTATGTAAAGAAATGTGGTGTTTCATCAGGGCAACAGTAATCACGGCAAATTATTCAT
AACAAAATGTGTTCAGCAGATTCAGTTAAAGTAGACTTATAAGTTACACAGTAACAAT
TCATCTGCTCAGCCTCATTTTGAAGTAGATAAAATATATTTTATTAGGAAACTCTGGG
GAGATATAAGGGAAAGCTTGCCTAAAAGTAGATGTTCTGTATATTATTTGGTAGTCAA
AGATGATTTCATGAAAAAAGGTTATTTGTAAAAAGTACAAAATGGGTAGAGACTAGAC
AATAAAAAGTAAGGAGTAAAAAACTAGGTATGTAACGTATATTAAAATAATTTTATGA
TTTTAATATTTACTGCACATTTTCTACAGTGCAGTGATTTGTATAACCATGCAATTAT
CAAATGCTTAGTGCCTTCACACAAAGTGCCTTTAATAAAAATTATTTTATAAATTATC
ATATTTTCTTTATATGTAGTCATCATCTTTTTTGTCTCATTTCTTGGAATCGTTCTAC
TTATGTTCTACTGATATGTTTTTTACCCGAGACCTATCTTGTCCTCTAAAGTAATTGG
CTTGTCAACTGGCTGTAGGGGGATTTTCAGAGTTATAGCTTAGTACTGTTAATGAGCC
ATAGGTTGAAATAGTGCTCTAGATTTACATGTTGTACAACAGTTATTGCAATATGTGT
AGGGGGGGGG

TABLE 8 - XSARA2

MPKMVIGDTDMAEDSLFNTGPSEIVCNSIVESQSLEVLDDVPVSINNEKSVLLDDGFS
PYSSPKSVLNSACLTMNNGKPSHGQKIVNDQDKEAVTISVLPMIIQDTTNVSTDPAFN
KSGTEEAYSALKQTTSVILPEIKPYSIQAALSCENINKIPRCQLNNTDLLSISPVVEA
CSEKQQNHTTSLHEKKLAAVSATAFFPVTAAETVLGNEALHSADFFDIVVKNVSDSCV
FNGDLTRTNGLSQENNEMFYASKELEGGVDANILLEDACIAYKERIDLSEENGTNAPM
YLYNGCDSYGMKNPAVRQNPKNLPSKEDSVTEEKEIEESKSEYYSGVYEQQKEDDITE
RGGVLLNAKVDQMKNSLHSLYNPVPSMHGQTSPKKGKIVQSLSVPYGGARPKQPTHLK
LNIPQPLSEMLQCDLIPPNAGCSSKNKNDMLNKSNRGDNLISESLREEVHSPVTDTNG
EVPRENRGPGSLCLAVSPDSPDNDLLAGQFGVPISKPFTTLGDVAPVWVPDSQAPNCM
KCEARFTFTKRRHHCRACGKVCKEMWCFIRATVITANYS

TABLE 9

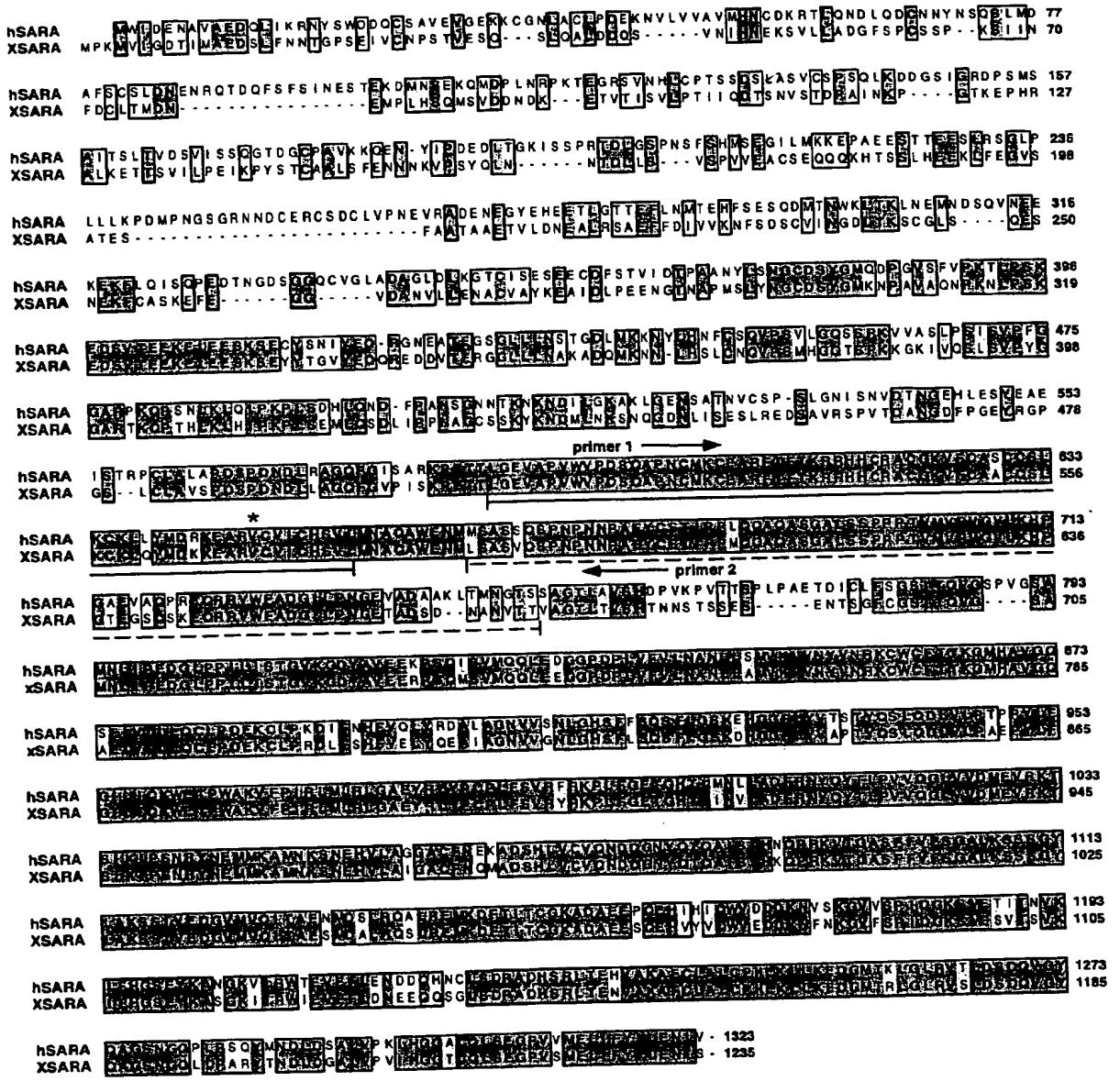
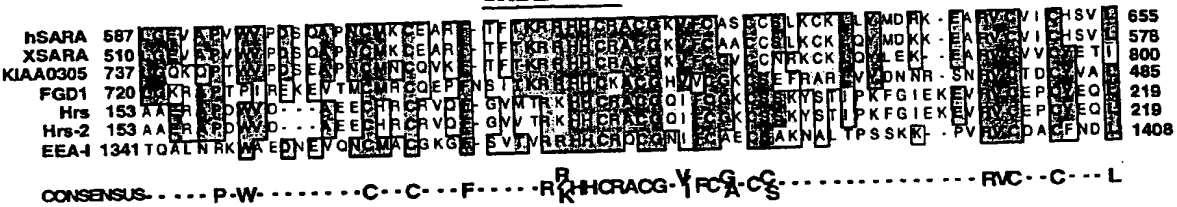


TABLE 10



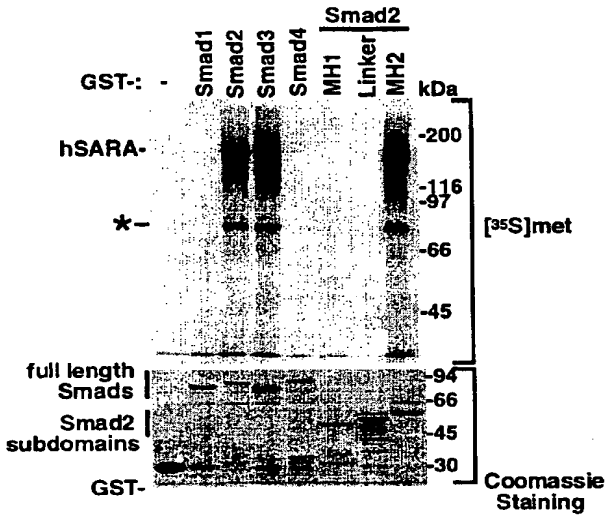

Smad2
Smad1
Smad2
Smad3
Smad4
MH1
Linker
MH2
GST-: -
kDa
hSARA-
-200
-116
-97
*–
-66
[35S]met
-45
full length Smads
-94
-66
Smad2 subdomains
-45
-30
GST-
Coomassie Staining

FIGURE 1

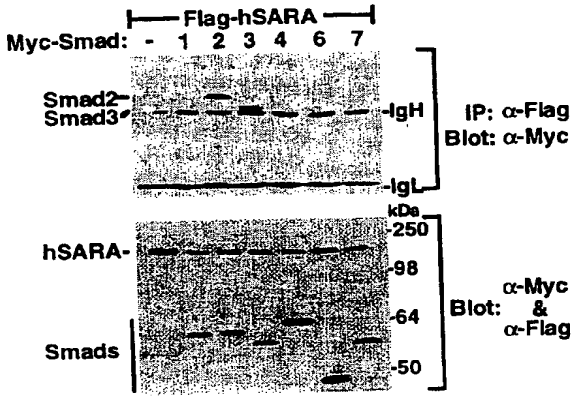

Flag-hSARA
Myc-Smad: - 1 2 3 4 6 7
Smad2
Smad3
IgH
IgL
IP: α-Flag
Blot: α-Myc
kDa
250
98
64
50
hSARA
Smads
Blot: α-Myc & α-Flag

FIGURE 2

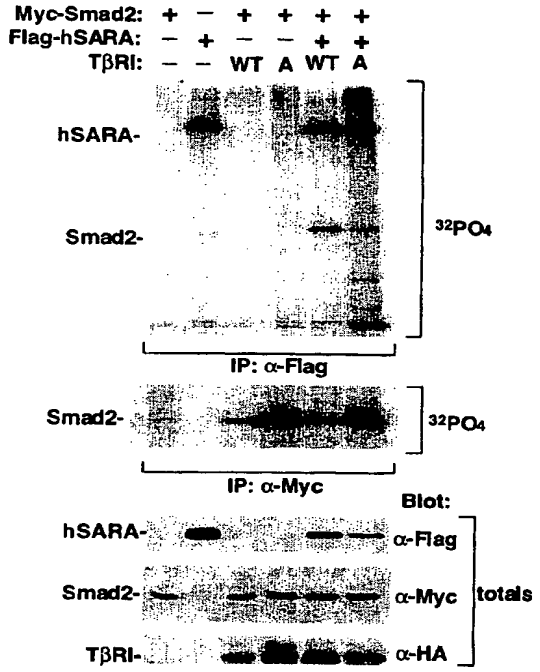

Myc-Smad2: + − + + + +
Flag-hSARA: − + − − + +
TβRI: − − WT A WT A
hSARA-
Smad2-
32PO4
IP: α-Flag
Smad2-
32PO4
IP: α-Myc
Blot:
hSARA-
α-Flag
Smad2-
α-Myc
totals
TβRI-
α-HA

FIGURE 3

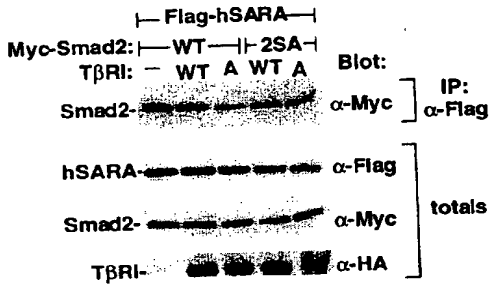

Flag-hSARA
Myc-Smad2:
WT
2SA
TβRI:
WT A WT A
Blot:
Smad2-
α-Myc
IP:
α-Flag
hSARA-
α-Flag
Smad2-
α-Myc
TβRI-
α-HA
totals

FIGURE 4

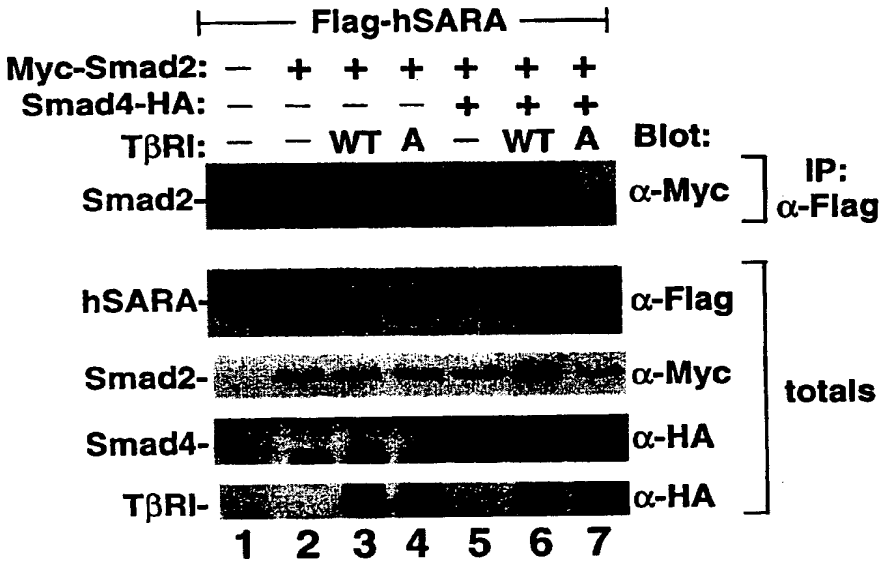

Flag-hSARA
Myc-Smad2: – + + + + + +
Smad4-HA: – – – – + + +
TβRI: – – WT A – WT A
Blot:
Smad2-
α-Myc
IP:
α-Flag
hSARA-
α-Flag
Smad2-
α-Myc
totals
Smad4-
α-HA
TβRI-
α-HA
1 2 3 4 5 6 7

FIGURE 5

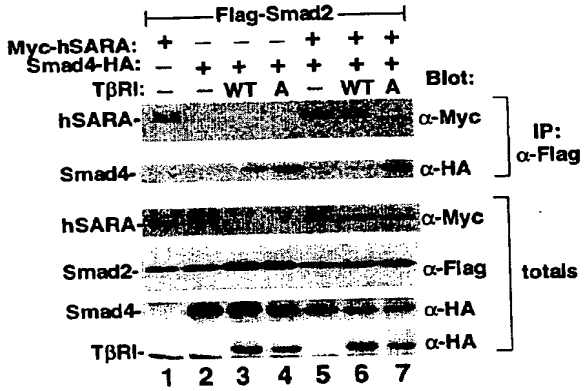

Flag-Smad2
Myc-hSARA: + – – – + + +
Smad4-HA: – + + + + + +
TβRI: – – WT A – WT A
Blot:
hSARA-
α-Myc
IP:
α-Flag
Smad4-
α-HA
hSARA-
α-Myc
Smad2-
α-Flag
totals
Smad4-
α-HA
TβRI-
α-HA
1 2 3 4 5 6 7

FIGURE 6

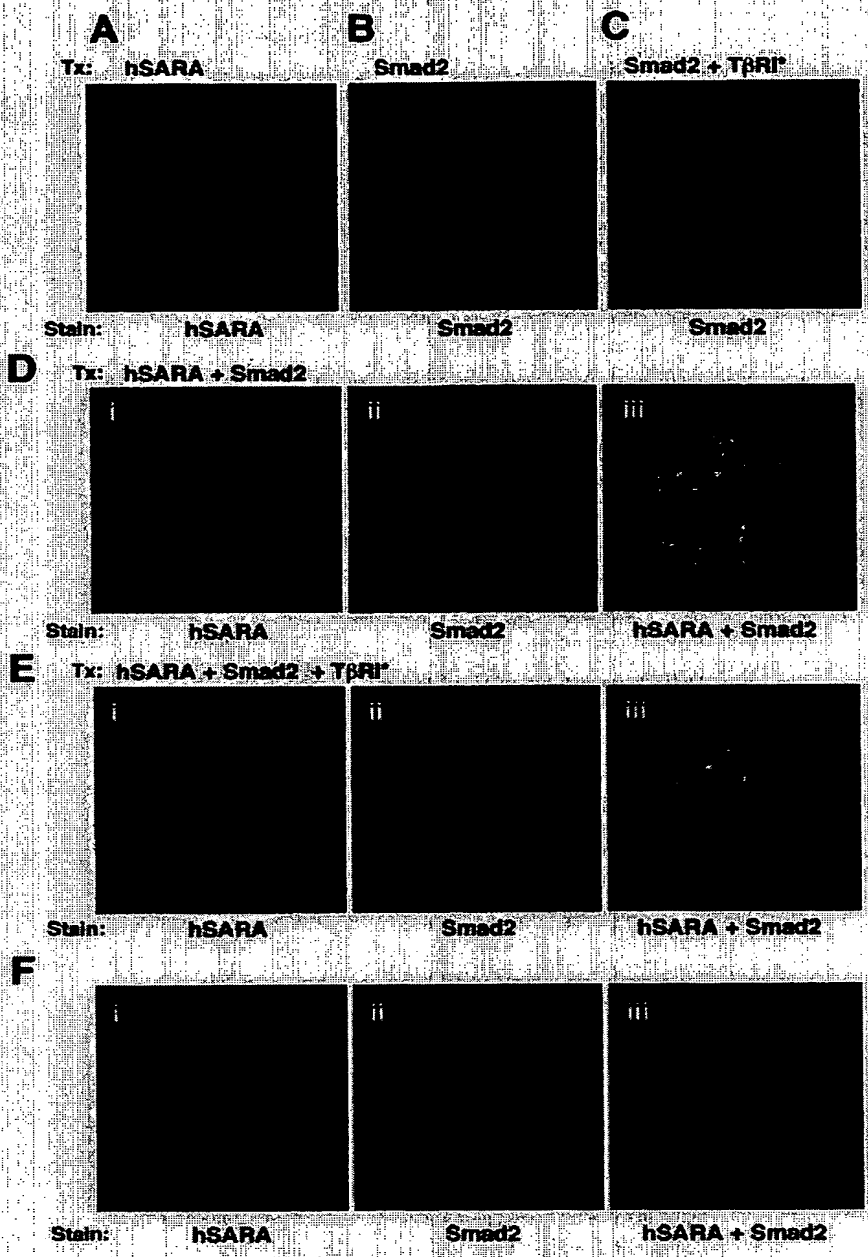


FIGURE 7

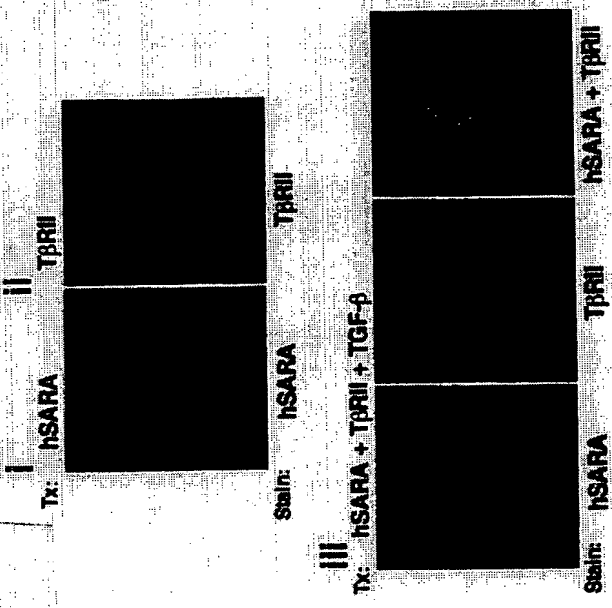


FIGURE 8A

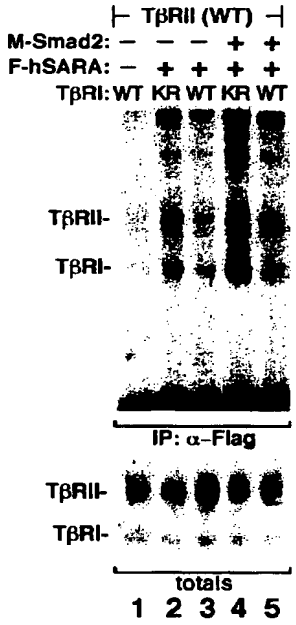

TβRII (WT)
M-Smad2: – – – + +
F-hSARA: – + + + +
TβRI: WT KR WT KR WT
TβRII-
TβRI-
IP: α-Flag
TβRII-
TβRI-
totals
1 2 3 4 5

FIGURE 8B

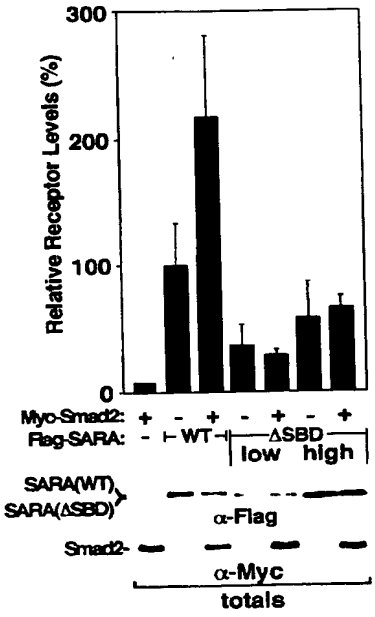

300
200
100
0
Relative Receptor Levels (%)
Myc-Smad2: + - + - + - +
Flag-SARA: - WT ΔSBD
low high
SARA(WT)
SARA(ΔSBD)
α-Flag
Smad2-
α-Myc
totals

FIGURE 9A

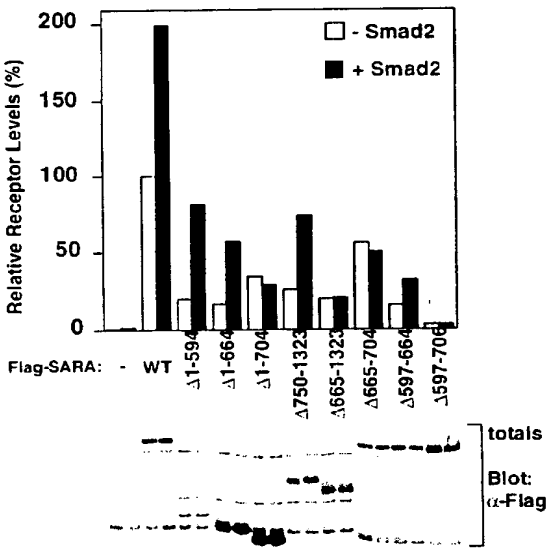

200
150
100
50
0
Relative Receptor Levels (%)
- Smad2
+ Smad2
Flag-SARA: - WT Δ1-594 Δ1-664 Δ1-704 Δ750-1323 Δ665-1323 Δ665-704 Δ597-664 Δ597-706
totals
Blot:
α-Flag

FIGURE 9B

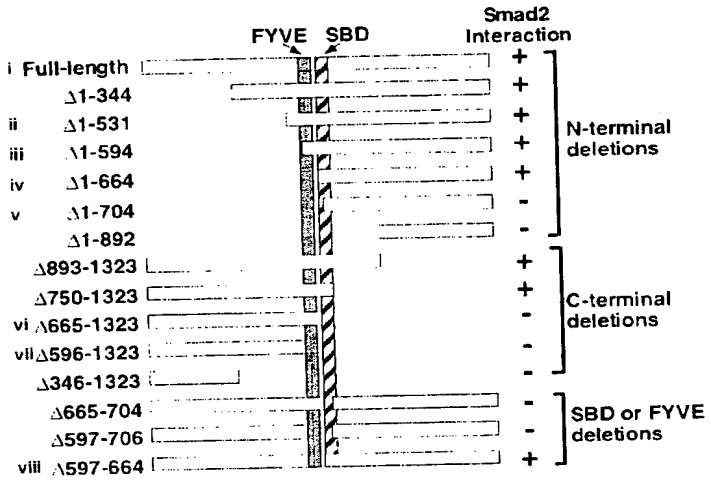

FYVE
SBD
Smad2 Interaction
i Full-length
Δ1-344
ii Δ1-531
iii Δ1-594
iv Δ1-664
v Δ1-704
Δ1-892
Δ893-1323
Δ750-1323
vi Δ665-1323
vii Δ596-1323
Δ346-1323
Δ665-704
Δ597-706
viii Δ597-664
+
+
+
+
+
-
-
+
+
-
-
-
-
-
+
N-terminal deletions
C-terminal deletions
SBD or FYVE deletions

FIGURE 10

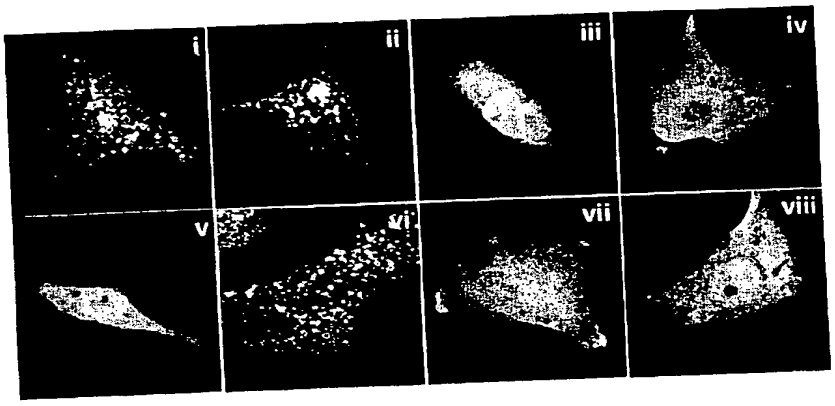


FIGURE 12

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1998-12-10

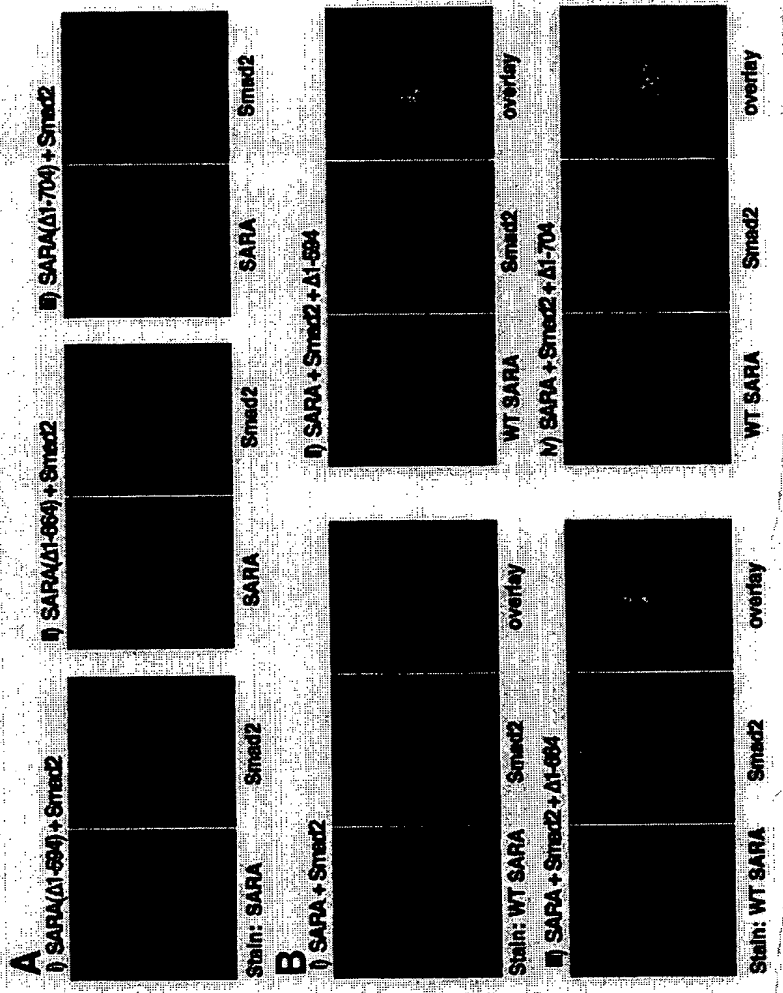

FIGURE 13

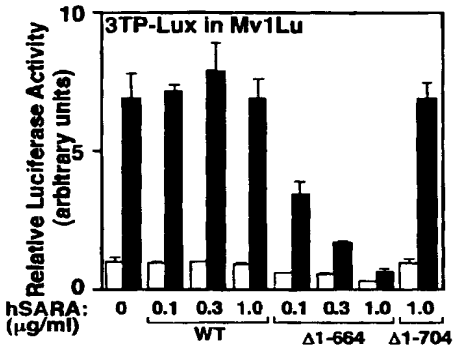
3TP-Lux in Mv1Lu
Relative Luciferase Activity (arbitrary units)
10
5
0
hSARA: (μg/ml)
0
0.1
0.3
1.0
0.1
0.3
1.0
1.0
WT
Δ1-664
Δ1-704

FIGURE 14

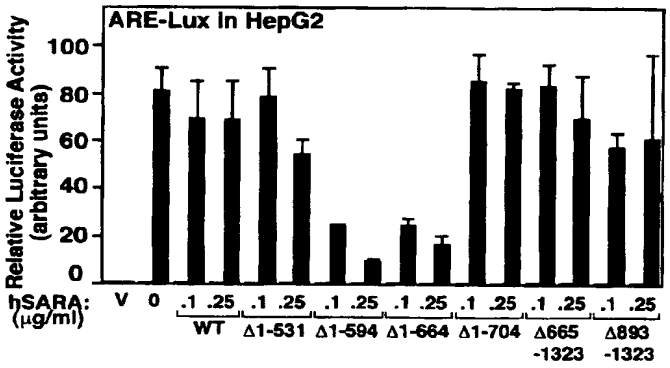
ARE-Lux in HepG2
Relative Luciferase Activity (arbitrary units)
100
80
60
40
20
0
hSARA: (μg/ml)
V 0 .1 .25 .1 .25 .1 .25 .1 .25 .1 .25 .1 .25 .1 .25
WT
Δ1-531
Δ1-594
Δ1-664
Δ1-704
Δ665 -1323
Δ893 -1323

FIGURE 15

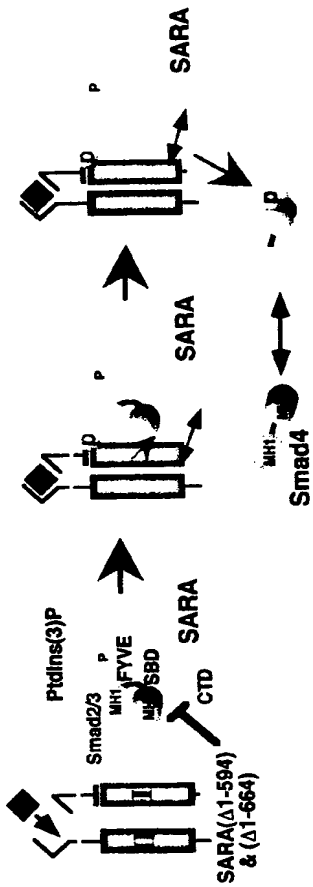
SARA
P
P
P
SARA
P
P
MH1
Smad4
Ptdins(3)P
P
Smad2/3
MH1
FYVE
SBD
SARA
CTD
SARA(Δ1-594)
& (Δ1-664)

FIGURE 18